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Physiological Zoölogy

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ON THE ROLE OF SEX HORMONES IN SEX DIFFERENTIATION IN THE OPOSSUM (DIDELPHYS VIRGINIANA)[1]

(Six plates)

CARL R. MOORE
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I. INTRODUCTION

IT IS still a current problem to define, even within broad limits, the forces that are operative in sexual differentiation. Although it is generally agreed that sex determination occurs at fertilization, it is well known that the embryos of each sex, during development, exhibit definite features that characterize both sexes; at a given stage in development the gonads may be indistinguishable from each other, and both Wolffian ducts (male components) and Müllerian ducts (female components) are present and

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[1] This investigation has been aided by grants from Armour and Company and from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

It is a great pleasure to acknowledge with a debt of gratitude the contribution of most valuable aid in this investigation by the following persons: to Mr. Clarence D. Cook, Lakeside, Michigan, for a supply of adult opossums, as well as females bearing pouch young; to Mr. C. B. Coursen, General Biological

equally developed, irrespective of the determined sex. Sexual differentiation subsequent to this "indifferent stage" may take a direct course toward maleness or femaleness or may take an intermediate course in the event of unusual environmental influences; we are now well aware of sex reversals induced at will in lower vertebrates by changes in the temperature of the developing medium (Witschi, 1939), by transplantations of heterologous gonads (Humphrey, 1929, 1935), and through parabiotic influences (Witschi, 1927; Burns, 1930, 1931). (For extended review of these phases see Willier, 1939, and Witschi, 1939.) In cattle twins (freemartin) evident humoral transmissions from males to females induce marked modifications in females, covering almost a complete range from female to male (Lillie, 1917; Willier, 1921; Bissonnette, 1924). It is plainly evident, therefore, that zygotic sex determination does not establish an irreversible predestination and that either sex, up to a certain period, is capable of some modification in the direction of the alternative sex. Under normal conditions some force, or set of forces, exercises a sex-differentiating role that gives clear expression to the dominant sex factors. But unsolved at the present time, are such problems as the period over which these forces operate, what limitations they have in producing ultimate effects, and what additional factors may play a role in sexual differentiation.

It is a general assumption that zygotic sex-determining factors act also as sex-differentiating factors, at least up to a stage when gonadal differentiation presumably assumes control. Gonads, therefore, would be dependent upon zygotic factors; but duct differentiation would depend upon substances secreted by the differentiating gonad rather than upon the same forces that operate to produce the distinctive gonad. In this respect an adjunct to sexual differentiation would appear in vertebrates that is not present in invertebrates, just as sex hormones from the gonads in the mature stage control reproductive systems in vertebrates and not in invertebrates.

One of the first and clearest cases indicating that hormones play the role of duct differentiators was the case of the freemartin described by Lillie (1917). The incontrovertible facts in the case were ingeniously interpreted by Lillie on the basis of the then accepted contentions with regard to hormone action, some portions of which require modification on the basis of more recently established facts.

Subsequent to the preparation of pure chemical substances that act in adults as hormones, some of which may be the true gonad hormone that controls the adult reproductive system, various attempts have been made to determine their effects upon embryonic sex differentiation and to put to a test the theory that sexual differentiation is dependent upon such substances (Dantchakoff; Kozelka and Gallagher; Willier, Gallagher, and Koch; Wolfe and Ginglinger; Raynaud, Greene, Burrill, and Ivy; Turner; Burns; Moore). It is to be admitted that, even if the conception is true that hormones secreted by the developing gonads are responsible for duct differentiation, there is still no assurance that the substances exercising a morphogenetic role in the embryo are the same as those operating on the functional system of the adult.

Supply Company, for females bearing pouch young and for adults, supplied at cost; to Dr. Dorothy Price for invaluable aid in treatments and in handling materials; to Miss Sally Johnson and Mrs. Suzanne Claussen for excellent microscopical preparations; to Drs. G. Stragnell and E. Schwenk, Schering Corporation, for testosterone ointment, *t*-propionate ointment, and oil solutions of progynon-B; to Dr. George Cartland, Upjohn Company, for gonadogen; to Dr. Donald Wonder, Cutter Laboratories, for gonadin; to Dr. Oliver Kamm, Parke Davis and Company, for theelin; to Mr. Kenji Toda for preparation of drawings and photomicrographs; and to Dr. Joseph Schwab for photographs of live young in the marsupial pouch (Figs. 1 and 2).

The writer's interest in this problem was aroused by a simple suggestion in 1917 by Professor Frank R. Lillie to consider making an attempt to produce a freemartin effect by experiment. Such efforts, begun at once, have led into many bypaths in the course of their developments; but the main problem has been reapproached repeatedly, the principal object being to subject the differentiating reproductive system to hormones of the opposite sex. Such attempts on my part have involved, in order, (1) testis transplantation into pregnant females, (2) parabiosis between normal males and pregnant females, (3) transplantation of gonads into embryos *in utero*, (4) transplantations of developing embryonic tissue into postnatal hosts of different hormonal states, (5) injections of testis extracts in oil into the amniotic cavity, and (6) injections of chemical androgens into pregnant mothers. Experiences with the latter project, which was begun in 1937 and which involved injection of testosterone propionate into pregnant rats (Moore, 1940), suggested the desirability of some other experimental animal. It developed very early that injections of *t*-propionate into pregnant female rats led to the death of a large proportion of embryos by the 20th day of gestation, the birth of dead or weakened embryos, delayed parturition, and faulty lactation. The fact that injections of pregnant mothers led to the death of some or all embryos in 60 per cent of the cases caused some concern as to whether any noted departures from the normal could be attributed to specific hormone action in the embryo or whether it might be due to abnormal development as a direct result of nonspecific placental injury. Confronted with this problem, an effort was made to obtain experimental materials not subject to the same unknown possibilities; and in February, 1938, opossum pouch young were obtained to study the suitability of this material. A preliminary account of the effects of sex hormones on sexual differentiation has been published (Moore, 1939), as well as a short account dealing with the care of captive animals, their breeding periods, and growth of young (Moore and Bodian, 1940). Entirely independently, and almost simultaneously, Burns (1939*b*, 1939*c*) published his preliminary findings from similar treatments on pouch-young opossums and has since published more detailed accounts of his experiments (1939*d*, 1939*e*).

The advantages of marsupials for such studies will be obvious at once. The opossum young at birth is sexually undifferentiated, possesses functional Wolffian ducts for approximately 2 weeks, shows beginning development of Müllerian ducts on the 3d day, and their contact with the urogenital sinus by the 10th day; the gonad becomes recognizable as testis or ovary on or about day 3. The young, firmly attached to nipples in the marsupial pouch, remain readily accessible for direct treatment rather than indirect treatment through the mother. Although they are postnatal young, their precocious development, in some respects, makes them available for direct treatment at a period in sexual differentiation that corresponds with fetal development in placental mammals.

It has been anticipated that a study of the effects of hormones applied to developing embryos may provide a logical basis for a re-examination of the thesis that sexual differentiation, especially that of the duct system, rests upon a directional humoral influence (hormone or hormones) provided by the developing gonad; however, one must not lose sight of the differentiating mechanism responsible for directing development of the gonad itself. Whether such an influence, zygotic in contrast to humoral, fails to operate on duct differentiation and whether such forces are supplanted by a newly produced humoral substance of gonadal origin are the essential questions of the main problem under investigation.

II. THE MATERIALS

In another place some experiences have been related in obtaining pouch-young opossums for experimental work (Moore and Bodian, 1940). Briefly, for present purposes, adult opossums confined in outdoor pens on the grounds of the Whitman Laboratory on the campus have delivered 20 litters whose birth was known within 24 hours, and from these litters 124 young were obtained (see Pl. I, Figs. 1 and 2). Some of these young were removed from the pouch periodically, carefully measured as to neck-rump length, head length, and weight. They provided a key series with which young of unknown delivery could be compared and their ages estimated with a high degree of accuracy. Seventeen females bearing pouch young were received through shipments by express from the states of Louisiana, Florida, Arkansas, Missouri, Illinois, and Michigan, the young in most cases less than 15 days of age. These females, likewise, provided 124 pouch young whose ages were estimated by comparison with known deliveries. From these 248 pouch young a normal series of 65 stages has been preserved in Bouin's fluid, and the reproductive tract prepared in serial section. The age range from day 1 to day 100 includes more than one-half of the stages during the first 30 days of development in the pouch. Of the 65 stages, constituting the normal untreated series, 21 are of known age, 44 of estimated age. Some stages are represented by a single animal; several stages by both a male and female, in many instances littermates; while some of the stages are represented by 3–5 different individuals. One definite age is occasionally represented by young from 5 different females.

In the hormone-treated series, no account is taken of the many young whose deaths occurred during treatment; estrogens, especially, have proved decidedly toxic, and records and preservations here considered account only for those contributing to the problem. Subcutaneous injections are fairly satisfactorily done subsequent to day 15; but, owing to the small size of the young (25–30 mm.), quantitative accuracy cannot be attained; some seepage occurs frequently from the injection site. Prior to day 15 hormones have been administered largely as a cream ointment applied over the entire surface of the body by means of a small brush; the hormone is readily absorbed through the skin (see Moore, Lamar, and Beck, 1938). It follows that the entire number of young in each pouch must be treated alike to prevent contamination by contact between embryos treated differently or between untreated ones in contact with treated.

Androgens.—A lanolin cream ointment carrying free testosterone or *t*-propionate, as well as testosterone propionate in sesame oil, has been employed. Animals preserved following treatment with androgens represent variable lengths of treatment as well as treatments during different age periods. A total of 35 androgen-treated young, preserved at ages from day 16 to day 83, consists of 16 young from known-age litters and 19 of estimated age; 15 males and 20 females receiving treatment have constituted the bulk of materials from which effects are depicted; but other single young, fairly accurately estimated as 8-month and 1-year specimens, have afforded supplementary material.

Estrogens.—Estradiol treatment by ointment has been the principal means by which estrogenic substances were applied, but in addition theelin in oil and Progynon-B have been injected subcutaneously. Several entire litters have been lost during treatment, because of toxic effects of estrogenic substances. Young are frequently found detached from the nipple and dead in the pouch or cage at the rate of 1–3 daily, whereas, the day previous all young appeared healthy. Decreasing the dosage has resulted in obtaining several young that have withstood treatment for sufficient periods to be instructive. Preserved young treated with estrogens consist of 25 specimens, of which 21 were of known age, 4 of estimated age. Preservation was done between ages of 12 and 88 days, and the group contains 10 males and 15 females.

Gonadotropic substance.—Gonadogen and gonadin, each representing the gonadotropic activity from the serum of pregnant mares, were used in the case of a few small litters, but the materials in this treatment have been less adequate than with sex hormones. Seven young were preserved between the ages of 17 and 38 days, all being from known-aged litters and consisting of 3 males and 4 females; in addition to these 1 male and 1 female of an estimated age of 100 days received treatments.

III. NORMAL PROGRESSION IN SEX-DUCT DIFFERENTIATION

INTRODUCTION

The adult reproductive systems of the opossum, male and female, are shown in Figures 3 and 4 (Pls. II and III). The special features of the male to which attention should be directed are the absence of seminal vesicles and the presence of the inclosed type of prostate gland; rather than a discrete, well-circumscribed prostate gland, this type consists of numerous branched, tortuous glands opening into the urogenital canal and confined within the sheath surrounding this structure. This gland in the opossum has been studied recently by Chase (1939), to whose work the reader is referred. The female system is peculiar because of the fact that the paired Müllerian ducts never fuse posteriorly to form the body of the uterus, but, rather, each Mullerian duct remains separate; it should be emphasized that, since Mullerian ducts fail to fuse in either sex, males never exhibit a uterus masculinus. The lower ends of the Müllerian ducts, posterior to the point of approximation and usual fusion in placental mammals in the genital cord, undergo a special development (along with horns of the urogenital sinus) to constitute the lateral vaginal canals—a peculiarity of the marsupial reproductive system. Whereas spermatozoa ascending through the urogenital canal ("middle" or "posterior vagina" [Hartman, 1923]) pass through the lateral vaginal canals into the uterus proper and on to the oviducts, at parturition marsupials develop a new birth canal by means of a rupture of the uterus at the cervix; a passage is formed through connective tissue that enters into the urogenital sinus, and the fetus thus does not pass through the lateral vaginal canals. This peculiar birth procedure was first described by Hill (1900) for *Dasyurus*.

To determine the effects of hormones upon sexual differentiation, it became necessary to establish the normal sequence in development for both males and females. The writer is unaware of a satisfactory account of the details of male development in the opossum, though much information has been added by the study of Chase (1939). For female differentiation the recent account of Baxter (1935), while highly satisfactory in most respects for Müllerian duct development, leaves out of account the Wolffian system or its derivatives, and for the purposes of this investigation these are very important. Furthermore, it is highly desirable to have at hand normal stages in the development of both sexes seriated as to age in the same manner as experimental embryos are seriated. This assures a knowledge of the condition of structures present at the time of beginning treatment. McCrady (1938) deals primarily with prenatal development, but a more recent study by McCrady (1940) treats the later development of the urogenital sinus and phallus. Since it is expected that a more detailed description of portions of normal differentiation will form the subject matter of other papers, expecially the development of gonads, only sufficient detail on normal stages will be sketchily presented here to provide a background of development for comparison with treated stages.

Preservation of normal young must be at random as to sex until about day 12, when a careful examination of the young attached to nipples in the pouch permits a selective removal of sexes. Males at this time begin to show a slight scrotal elevation anterior to the phallus (the male opossum possesses a scrotum located well anterior to the penis), and females exhibit a definite mammary-gland pattern in the developing pouch that is clearly diagnostic of sex; rudimentary nipple anlagen are less prominent on males. Covering the first 16 days of pouch development, the normal series upon which this account is based contains 40 pouch young, 17 of known date of birth and 23 of estimated age; the group includes 15 males and 14 females, and the remainder were undetermined as to sex. Subsequent to day 16, many stages are represented by littermate males and females (days 24, 32, 44, 67, 75, 84, and 100). Intermediate stages are represented both by isolated males and females, and in some cases the same age is represented by several young of the same sex. Often large litters yielded two or three successive stages for the normal series before the remainder were assigned for treatments with hormones. Where possible, males and females of the same litter were selectively reserved for treatment.

The opossum young at birth (see Pl. I, Fig. 1) is sexually undifferentiated, but by the 5th day in the pouch gonads are assuming definite testicular or ovarian characteristics. Both sexes possess well formed and functional Wolffian ducts, since a functional mesonephros is present until approximately day 15; subsequent to this period, progressive mesonephric degeneration occurs, and the metanephros gradually becomes the functional excretory organ.

Müllerian ducts are discernible in the 3-day pouch young. A fairly distinct ostium is continued, usually by 3–6 sections of an epithelial cord with barely visible lumen, and further continues posteriorly as a solid cord of cells approximately one-half the length of the mesonephros, where it is lost. Day 10 introduces the beginning of sexual dimorphism in duct development. Because of its urinary function, the mesonephric duct is similar in both sexes, but Müllerian ducts exhibit slight sex differences.

10TH DAY

A. *Males.*—The male opossum pouch young of 10 days postnatal development exhibits a testis with definite seminiferous cords, a developing rete cavity with cords passing toward the mesonephros, a fully functional mesonephric duct that enters the urogenital sinus, and Müllerian ducts. The diameter of the lumen of Wolffian ducts in serial sections varies in its course from 0.04 mm. to 0.06 mm.

The Müllerian system is visible in the 10-day male as a fairly distinct ostium, from which an epithelial cord with only an occasional tiny lumen runs through the duct portion of the mesonephros. Posterior to the mesonephros, the Müllerian cord passes more indistinctly toward the genital cord but cannot be followed to contact with the urogenital sinus epithelium.

B. *Females.*—Females at 10-day pouch development differ from males (aside from the recognizable ovary) by the fact that the Müllerian system of ducts exhibits somewhat better development. The ostium is more definite; a very small lumen (0.009 mm.) is present throughout the upper two-thirds of the mesonephros. Posterior to this region the nonluminate epithelial cord leaves the mesonephros, passes to the genital cord alongside the

Wolffian duct, and its cells can be traced to contact with the urogenital sinus epithelium at the point of entry of Wolffian ducts.

DAYS 15 AND 16

The 15th or 16th day of pouch development is a fairly important age for the introduction of a few cardinal changes. At this time the metanephros exhibits well-formed tubules and the organization of the glomeruli has progressed remarkably. The mesonephric glomeruli, especially at the anterior end, exhibit marked disorganization. The entire renal corpuscle is often hyaline in nature, and the tubules exhibit marked shrinkage and fragmentation. By this period the metanephric duct (ureter) is larger and exhibits a more definite entrance into the urogenital sinus than does the Wolffian duct. In short, the metanephros is gaining in ascendancy as the mesonephros declines, and it is probable that at this period both are active in excretory function.

A. *Males.*—Representing this stage of development are 2 males of day 14, 3 of day 15, and 4 of day 16. The testicle exhibits definite and well-formed seminiferous cords; the rete presents a distinguishable, though small, ramifying rete cavity; and nonluminate cords of cells from the mesonephros make contact with it.

1. Wolffian ducts: In each of the 9 males of these 3 days of development the Wolffian duct retains a well-formed lumen within the mesonephros (luminal diameters, 0.06–0.03 mm.) As the duct enters the genital cord, it becomes progressively reduced, and in all 9 pouch young the entrance into the cavity is approaching extinction; a definite entrance is still present, and the diameter of the lumen in the genital cord is reduced to 0.007–0.01 mm.

2. Mullerian ducts: The ostium, at this time, is either extinct or unrecognizable. In each of the 9 males the ducts are represented by solid cords of cells in the mesonephros; and in the majority the cord is visible posteriorly, just ventral to the Wolffian duct, passing in the direction of, but unrecognizable in the substance of, the genital cord. A lumen is scarcely recognizable at any point along the course of the cord in any of the 9 males.

3. Prostate: This stage of development marks the first appearance of the prostate anlage in male embryos; in female opossums a prostate anlage is never recognizable at this, or any stage; hence, the structure is not represented in normal females—a point of especial importance in this investigation. The two 14-day-old males do not show recognizable prostate proliferations from the urogenital sinus epithelium. Of the three 15-day males, two show definite beginnings of local bud proliferation representing the structure, whereas the third does not show it. Each of the four 16-day males shows a very definite anlage for the prostate, which appears as definite knobs or buds from the basal layer of the urogenital sinus epithelium (see Fig. 10 for 32-day male).

B. *Females.*—This period is represented by one 14-day female, two 16-day females, and a single 17-day female.

1. Wolffian ducts: The Wolffian ducts of the 14-day female and one of the 16-day females were similar to those present in the 9 males of equivalent age, in that a well-preserved duct still exists in the mesonephros with a lumen diameter of 0.04–0.06 mm., and this tapers in the genital cord to a barely perceptible entrance of the lumen into the urogenital sinus. The second 16-day female was similar, with the exception that the lumen of the duct was interrupted in the genital cord and failed to enter the urogenital sinus. In the 17-day female not only was the Wolffian duct lumen interrupted at the urogenital sinus but the cord stopped short of these structures.

2. Müllerian ducts: In females of this period the Müllerian ducts exhibit definite but very small lumina to the level of contact with urogenital sinus epithelium. At no stage in normal female development up to the 100th day is the lumen of Müllerian ducts traceable into the cavity of the urogenital sinus. Baxter (1935) has clearly demonstrated that the distal portion of the adult female tubes owes their origin to the horn of the urogenital sinus; and during this organization of the duct, connections with the urogenital sinus are represented by a solid epithelial mass.

C. *Résumé.*—These rather brief notations emphasize that Wolffian ducts at the 15- and 16-day stage of development are beginning to lose their luminal connections with the urogenital sinus in both sexes—an indication that their former definite connections are associated with excretory function and that no perceptible difference is seen between males and females. They likewise call to mind that the Müllerian ducts in males, as solid cords, are inferior to females and now fall short of the genital cords, whereas in females they are luminate and make contact with urogenital sinus epithelium. Further sexual differences are a definite prostate anlage in males and an entire absence of this structure in females.

DAY 24 IN THE POUCH

For the purpose of emphasizing the beginning sexual dimorphism indicated as of day 15, attention should be directed to this progress a few days later, and for that purpose attention will be called to a littermate male and female removed from the pouch on day 24.

A. *The male.*—The testis is now better organized, with well-developed solid seminiferous cords. The rete is represented by a heavy mass of epithelial cells penetrating among the seminiferous cords in the testis and making contact with these. At the opposite end mesonephric tubules, some with tiny cavities representing lumina, are traced to contact with the rete. The rete cavity is definite though not of great extent.

1. Wolffian ducts: At this stage the Wolffian ducts are appreciably reduced in size in the substance of the mesonephros and retain lumina with the greatest diameter 0.03 mm., or approximately one-half that of the 15-day stage. The mesonephros is now almost totally devoid of Malpighian corpuscles, and mesonephric tubules are, for the most part, absent or atrophic. Posterior to the remnant of the mesonephros the small cells of the Wolffian duct are reduced to a solid cord for some distance anterior to the urogenital sinus. This active regression of the Wolffian duct in the normal male, subsequent to the loss of function as a urinary duct, suggests that its differentiation or maintenance up to this period is not conditioned by hormone secreted by the testis.

2. Müllerian ducts: These are small nonluminate cords in the region of the degenerating mesonephros and no longer extend into the genital cord. The atrophy of these ducts in normal males is from the posterior toward the anterior end.

The prostate gland is much advanced in its development since the 15-day stage. It is represented by numerous large buds and epithelial knobs growing out from the urogenital sinus epithelium (see Pl. V, Fig. 10, for prostate in 32-day male).

B. *The female.*—

1. Wolffian duct: In normal 24-day females the Wolffian duct is reduced to a solid cord of cells throughout most of its extent, but in a few sections located at the gonad level a very small lumen is present. This small cord of cells is lost anterior to the urogenital sinus.

2. Müllerian duct: The Müllerian duct of females is a well-formed tube composed of a relatively thick epithelium and contains a lumen through most of its course. At the anterior end the lumen is very small, but more posteriorly it shows a slight enlargement indicative of the future uterine portion. In the genital cord the two tubes approach closely in the mid-ventral line at the region known as the cervix (Pl. III, Fig. 4); fusion, however, does not occur in the opossum female at any stage of development. Posterior to the cervix region, the tubes become decidedly more separated and begin to show a slight, though definite, enlargement for the lateral vaginal canals. The lumen of this portion is further traceable toward contact with the urogenital sinus; but, as is usual, this portion is represented by a solid mass of cells continuous with the sinus horn epithelium.

Thus, by the 24-day stage the female tract is marked off into its future parts of oviduct, uterus, cervix, and lateral vaginal canals. Figure 5 (Pl. IV) reveals the dissected female system at 31 days; and Figure 7 (Pl. V) reveals sections of the cervix and uterine level of the Müllerian duct.

Cowper's gland, which arises in both sexes at approximately day 9 as outgrowths from the distal portion of the urogenital sinus epithelium, appears somewhat further developed in the male of the 24-day stage than in the female. In each case the anlage is still a solid cord of cells extending away from its point of origin and beginning to show branchings.

44TH DAY

A. *The male.*—The testes of 44-day males are quite characteristic of inactive testes whose seminiferous cords are distinct and contain layers of cells shifting peripherally and suggesting a stage prior to the development of a lumen; no lumen is yet evident, however, and none will appear until the animal approaches the breeding season of the year following birth.

The epididymis in section consists of well-defined cords, some of which exhibit a definite lumen. The tubules can be traced to contact with the rete testis, which now shows a well-defined rete cavity at the position of the future hilus of the organ. It appears that a single tubule from the epididymis makes contact with the rete cavity, but this becomes subdivided into several epididymal tubules outside the testis.

The vas deferens is traceable as a tube from the epididymis to within a few sections anterior to its contact with the epithelium of the urogenital sinus; no luminal connection exists between the duct and the sinus cavity. Diameters of the lumen range from 0.01 to 0.03 mm.

Müllerian ducts are reduced to solid cords of epithelial cells from a point immediately below the epididymis to approximately one-half the length of the vas deferens, beyond which it is no longer recognizable.

The prostate gland is represented by well-circumscribed solid cords of cells growing out from all sides of the urogenital sinus epithelium, some showing definite branching.

B. *The female.*—The ovary at 44 days has advanced in development and is quite different, in detail, from the 24-day stage. The epoöphoron has exhibited marked development; and, whereas it is somewhat smaller as regards number of cross-sections of tubules, these are approximately in the same grade of development as those of the male; cords are traceable through the mesovarium to the somewhat enlarged rete cavity located within the developing medulla, surrounded by a cortex that exhibits decided activity.

The Wolffian ducts of the female are reduced to nonluminate cords of poorly organized cells, which continue, along with the Müllerian ducts, approximately two-thirds the distance to the genital cord.

Müllerian ducts have progressed to the point of very definite differentiation of parts—oviducts, uterus, cervix, and lateral vaginal canals. Oviducts have become tortuous in their course and show a greatest luminal diameter of 0.03 mm. The uterine portion is decidedly expanded and exhibits a luminal diameter up to 0.30 mm. The epithelium is yet simple, and no glandular outgrowths are evident. The constricted cervical portion of the tube is narrowed to a diameter of 0.07 mm. and continues into the lateral vaginal canals, which are also expanded until their luminal diameters reach 0.37 mm. Posteriorly the tubes continue to contact with the urogenital sinus epithelium, the posterior portion being composed of a solid epithelial cord mass.

Cowper's gland exhibits marked differences in the two sexes at this stage. In the female this structure consists of well-formed heavy solid cords growing out from the urogenital sinus epithelium. In the male the outgrowth is approximately twice as large, shows more marked branchings, and is luminate.

DEVELOPMENT UP TO 100 DAYS

It will suffice, for the present account, to outline in a brief manner the further development of the normal reproductive system up to the 100th day, which will largely cover the ages subjected to artificial hormonal influences.

A. *Males.*—Testis differentiation continues slowly up to the 100th day, at which time the seminiferous tubules consist essentially of a single basal layer of cells representing the germinal epithelium but no development of tubular lumina. In 8-month-old males seminiferous tubules are luminate, and the germinal epithelium is actively producing sperm heads and spermatozoa; 1-year animals show greater quantities of spermatozoa.

The vas deferens between the 44th and 100th day increases gradually in size and in the differentiation of its walls; the greatest luminal diameter of 0.03 mm. at day 44 reaches 0.04 mm. by day 67, 0.06 by days 83–100. The urogenital sinus end is consistently closed by a solid epithelial mass representing the urogenital-sinus-horn portion of the duct; at day 100 the lumen is traceable into the cavity of the urogenital sinus.

Prostate-gland development involves a gradual increase in size of the entire mass of the urogenital sinus, with the prostatic outgrowths becoming more profusely branched: these outgrowths from the sinus epithelium begin to exhibit slight cavities at their medial ends by day 83. Growth indications were recorded as diameters of the structure between the developing muscle layers, rather than total outside diameter, in order to portray, as nearly as possible, prostatic growth. These diameters have increased from 0.5 mm. on day 40 to 1.0 mm. on day 75, 1.5 mm. on day 100, 2.7 mm. at 8 months, and 9.0 mm. at 1 year. The external character of the adult prostate will be seen from Figure 3 (Pl. II), and a section of a 100-day prostate in Figure 14 (Pl. V). A section of the prostate of the 32-day stage is shown in Figure 10 (Pl. V).

Müllerian ducts in males, which at day 44 were represented by a solid cord of cells extending approximately one-half the distance from the epididymis to the urogenital sinus, show increased growth activities in the remnants subsequent to this period. On day 67 the anterior portion of this cord displays a lumen having a diameter of 0.01 mm. This continues its development until, on day 83, the entire remaining portion of the persisting cord contained a lumen of 0.01–0.03 mm. A similar condition holds on day 100 with a diameter of 0.04, and in one specimen a dilated portion measured 0.11 mm. Each end of the tube located along the developing epididymis was closed.

B. *Female.*—Ovarian development involves many changes in its final differentiation, the subject of a future communication; but by day 100 well-developed follicles containing large ova are present; no antrum-bearing follicles appear even at the 8-month stage of development. Peculiar syncytial masses, formerly making up the principal portion of the cortex area and designating an incomplete development, are still evident at day 100.

The epoöphoron continues its development subsequent to day 44 and reaches relatively large proportions in the opossum female. Connections exist between the cords or tubules of the epoöphoron and the rete ovarii, and the latter exhibits a relatively large cavity within the ovarian medulla of the opossum ovary. Epoöphoron tubules also connect with the Wolffian duct, which remains at the 100th day in the upper portion of its course; and, whereas the duct is devoid of a lumen on day 44, growth and further differentiation involve the gradual reformation of a lumen in the 100-day female of 0.09 mm. in diameter.

The Müllerian system continues its development from day 44 by increases in the folds of the oviducts, by uterine enlargement, and by enlargement of the lateral vaginal canals. Oviducal lumina increase from 0.03 mm. on day 44 to 0.11 mm. on day 100.

Uterine development by day 100 (Pl. VI, Fig. 22) involves gradual increases in luminal diameters from 0.30 mm. on day 44 to 1.05 mm. Glandular outgrowths later make their appearance as proliferations of the lining epithelium; and by the 8-month stage the uterus exhibits a much vacuolated, cheesy type of development due to the tortuosity of the glands.

Lateral vaginal canals have similarly increased perceptibly in size and differentiation and are demarcated from the lower uterus by the narrow cervical portion. The lumen of the lateral vaginal canal attains a diameter of 0.90 mm. by day 100, whereas that of the cervix is 0.07 mm.

Cowper's gland in 100-day males and in females has continued its differentiation as a result of further development of epithelial buds, their branching, and the appearance of cavities within the local portions.

IV. EFFECTS OF ANDROGENIC TREATMENTS

Androgens have been administered daily to male and female pouch young as an ointment of testosterone and of *t*-propionate and by subcutaneous injection of *t*-propionate in sesame oil. Treatments prior to day 15 have usually been by ointment, owing principally to the difficulty of injecting very small animals, in a few cases hormones in alcoholic solution were applied on the skin, but this treatment was discontinued because of the greater loss of young from the pouch. Some young have been injected subcutaneously by the 10th day, when the total body length was 20 25 mm. and the total weight 0.8 gm., but injections subsequent to day 16 were more satisfactory; as the animals grew, injections naturally became progressively simpler. Dosages by inunction have been variable and are difficult to approximate with any precision, since greater quantities of ointment can be applied as the animal increases in size. Subcutaneous injections prior to day 70 have been given in total daily doses of 0.25 mg. of *t*-propionate in 0.05 cc. of sesame oil, but unavoidable seepage from the skin puncture occurred frequently. Subsequent to day 70, total daily doses have been 0.5 mg. in some cases and 5.0 mg. in others.

Without consideration of animals lost during treatment, 18 males have been preserved at ages from 16 to 100 days, and several additional ones were treated at ages of 8 months and 1 year. Twenty-two androgen-treated females were preserved at the close of treatment. From this group of 40 preserved, androgen-treated animals, in addition to still older ones, 13 males and 16 females have been selected for brief tabulation in Table 1. This table shows types of treatment, age at preservation, and responses represented by ocular micrometer measurements of structures from serial sections, as well as subjective indications of relative response of structures. Selection for tabulation was made to include animals treated by the three methods of hormone administration, and in particular to include littermate males and females. Males and females in the table designated by the same number (i.e., ♂ 96A, ♀ 96B) are in every case littermates.

A. *Effects on males.*—The most outstanding effect of androgenic treatment on pouch young has been the stimulation of the external genitalia and of the prostate gland and of Cowper's gland; since the latter structure will be considered in more detail in another place, it will receive only brief mention here. Slight stimulation only has been seen on Wolffian-duct and Müllerian-duct development in males. No inhibitory effects have been noted in any case. Attention is here called to Table 1 for a brief notation of responses.

1. Wolffian duct: Responses of the Wolffian duct in androgen-treated males have been surprisingly minimal. It should be recalled that in normal males definite but partial Wolffian duct involution follows loss of the urinary function of the mesonephros. The diameter of the lumen of this duct decreases from a maximal of 0.09 mm. on the 12th–14th days to approximately 0.04 mm. from days 16–18 up to about day 83. The lumen of the duct can be traced into the urogenital sinus up to approximately day 16; but, subsequent to this period, the duct entrance is closed by a solid epithelial cell mass, associated apparently with modifications of the urogenital sinus horns with which they connect (see Baxter, 1935). Indications given in this table suggest that in 3 of the 12 treated males there was a slight stimulation of the Wolffian duct; judgment of such stimulation was based largely on the condition of the duct and the size of the lumen in treated males compared with normal males of similar age. Such larger luminal diameters are frequently fairly localized and fail to extend over the entire course of the duct. It is to be emphasized that in no case did treatment with androgenic substances prevent the reduction in size of the duct subsequent to the loss of its urinary function, even though treatment was begun many days prior to mesonephric degeneration. A section of the Wolffian duct (vas deferens) of the 31-day male 64A, treated with *t*-propionate by subcutaneous injections from day 10 to day 30, can be seen in Figure 9 (Pl. V): it does not reveal stimulation, in contrast to a much stimulated prostate (compare prostate with normal ♂, Pl. V, Fig. 10) and the greatly stimulated external genitalia. In a similar manner androgenic treatment failed to maintain luminal connection of the duct with the cavity of the urogenital sinus. At the age of 83–100 days, treatment appears to hasten the re-establishment of luminal connections with the sinus, but the evidence is considered none too definite.

In contrast to the duct (vas deferens), the epididymal tubules (mesonephric) appear to respond to androgens, exhibiting more tortuous tubules, better lumina, and better tubule walls. No comparative measure has been found entirely satisfactory to express these differences; hence, they must be based upon subjective judgment.

2. Müllerian duct: The Müllerian duct in males responded more definitely to androgenic treatment than did Wolffian ducts, but this may be associated with a much greater involution of the former. To recall the condition in normal males, the Müllerian duct

TABLE 1

EFFECTS OF ANDROGENS ON OPOSSUM YOUNG*

SER. No.	AGE AT DEATH (DAYS)	TREATMENT (DAYS)	WOLFFIAN DUCT	WOLFFIAN-DUCT STIMULATION	PROSTATE UROGENITAL SINUS	PROSTATE STIMULATION	MÜLLERIAN DUCT					PHALLUS
							Oviduct	Uterus	Cervix	Lateral Vaginal Canal	Stimulation	
	Males											
96A..	16	3–15 TO	0 03 (0 06)	0	0 60 (0 60)	+		0 00 (0.00)			0	2 2×1 2
63A	22	10–21 TPI	0 04 (0 03)	0	0 90 (0 63)	+		0 01 (0 00)			?	2 0×1 8
47A..	24	3–24 TO	0 02 (0 03)	0	1 05 (0 63)	+		0 00 (0.00)			0	3 0×2 3
38B..	25	15–25 TPO	0 03 (0 03)	0	0 90 (0 63)			0 00 (0 00)			0	2 1×1 5
27A. .	27	16–27 TO	0 07 (0 03)	+	0 75 (0 63)	+		0 00 (0 00)			0	....
101A.	30	3–15 TO 16–29 TPI	0 09 (0 03)	+	1 42 (1 05)	++		0 01 (0 00)			+	3 0×2.1
64A.	31	10–30 TPI	0 03 (0 03)	0	1 27 (1 05)	++		0 03 (0 00)			+	3 1×2.5
31A	32	16–31 TO	0 04 (0 03)	0	0 90 (1 05)	+		0 01 (0 00)			?	3 0×2 0
30A.	32	16–31 TPI	0 11 (0 03)	+	1 12 (1 05)	++		0 04 (0 00)			+	3 0×2 2
43B.	32	16–31 TPO	0 03 (0 03)	0	1 12 (1 05)	+		0 00 (0 00)			0	2 8×2.0
65A.	39	10–31 TPI 32–39 no tr	0 06 (0 03)	0	1 42 (1 05)	+++		0 09 (0 00)			+	3 7×3 0
92...	83	70–82 TPI	0 04 (0 06)	0	2 85 (1 35)	+++		0 03 (0 03)			0	7 5×3 5
98..	100	70–99 TPI	0 07 (0 07)	0	4 9 (1 50)	++++		0 03 (0 05)			0	15 5×6.0

* Phallic measurements from 80 per cent alcohol before sectioning Prostatic measurements, greatest diameter of cross-section of urogenital sinus and prostate outgrowths. Other measurements, maximal diameter of duct lumina (ocular micrometer measurements from sections). All measurements expressed in millimeters Figures in parentheses represent normal embryos Stimulation. +, slight, ++, moderate; +++, great; ++++, extreme, 0, no effect; no tr., no treatment; TO, testosterone ointment; TPO, *t*-propionate ointment; TPI, *t*-propionate injection.

TABLE 1—*Continued*

SER. No.	AGE AT DEATH (DAYS)	TREATMENT (DAYS)	WOLFFIAN DUCT	WOLFFIAN-DUCT STIMULATION	PROSTATE UROGENITAL SINUS	PROSTATE STIMULATION	MÜLLERIAN DUCT					PHALLUS
							Oviduct	Uterus	Cervix	Lateral Vaginal Canal	Stimulation	
Females												
96B.	16	3–15 TO	0 03 (0 06)	0	0 48 (0 60)	+		0 03 (0 00)	.	.	+	2 2×1 2
63B .	22	10–21 TPI	0 04 (0 00)	+	1 12 (0 75)	++	0 03 (0 00)	0 49 (0 01)	0 07	1 05 (0 01)	+++	1 8×1 4
47B.	24	3–24 TO	0 06 (0 00)	++	0 90 (0 75)	+++	0 c3 (0 00)	0 11 (0 01)	0 03	0 30 (0 01)	++	2 2×1 8
38A .	25	15–25 TPO	0 00 (0 00)	0	0 90 (0 75)	+	0 03 (0 00)	0 06 (0 01)	0 03	0 12 (0 01)	+	1 7×1 2
27B .	27	16–27 TO	0 06 (0 00)	+	0 75 (0 75)	++	0 01 (0 00)	0 09 (0 01)	0 02	0 07 (0 01)	+	. ....
101B .	30	3–15 TO 16–29 TPI	0 11 (0 00)	++	1 20 (1 05)	+++	0 07 (0 03)	0 65 (0 07)	0 06	1 42 (0 11)	+++	4 0×2.5
64B ..	31	10–30 TPI	0 26 (0 00)	+	1 42 (1 05)	+++	0 03 (0 03)	1 35 (0 07)	0 03	2 70 (0 11)	++++	3 0×2.5
31B .	32	16–31 TO	0 04 (0 00)	+	0 90 (1 05)	++	0 03 (0 03)	0 18 (0 07)	0 03	0 26 (0 11)	++	2 4×1.8
30B .	32	16–31 TPI	0 05 (0 00)	+	1 27 (1 05)	+++	0 07 (0 03)	0 30 (0 07)	0 03	1 42 (0 11)	+++	3 1×2 1
43A .	32	16–31 TPO	0 03 (0 00)	+	1 12 (1 05)	++	0 03 (0 03)	0 33 (0 07)	0 03	0 57 (0 11)	++	2 0×1 8
65B	39	10–31 TPI 32–39 no tr.	0 37 (0 00)	+	1 50 (1 20)	++++	0 05 (0 03)	1 50 (0 30)	0 02	1 60 (0 37)	+++	3 0×2 7
107.....	45	3–15 TO 16–44 TPI	0 07 (0 00)	++	1 95 (1 20)	+++	0 04 (0 03)	0 22 (0 30)	0 03	0 52 (0 37)	+++	4 6×3 0
66.	62	10–31 TPI 32–62 no tr.	0 75 (0 00)	++	2 25 (1 20)	++++	0 07 (0 03)	1 65 (0 60)	0 04	0 60 (0 90)	+++	3.0×3.2
117.. .	95	3–15 TO 16–93 TPI	0 02 (0 00)	+	5 50 (1 35)	++++	0 03 (0 03)	0 82 (1 05)	0 07	0 90 (0 90)	++	7 8×5 5
118.	95	3–44 TPI 45–95 no tr.	0 37 (0 00)	++	2 10 (1 35)	++++	0 04 (0 04)	0 30 (1 05)	0 07	1 50 (0 90)	+++	5.2×1.8
120.....	147	3–95 TPI 96–147 no tr.	0 70 (0 00)	++	3 60 ( )	++++	0 05 ( )	0 82 ( )	0 07	0 90 (. .)	+++	9 6×5 5

appears on day 3, gradually extends posteriorly as a solid cord of cells which reaches the genital cord on days 8–10; but the duct fails to make contact with the urogenital sinus epithelium. By days 12–14 the solid cord of cells is largely restricted to the mesonephros level, or slightly posterior to it, and is not usually evident in the genital cord. Throughout its course the Müllerian duct remains as a solid cord in the male, becoming progressively shorter until approximately day 50, when the anterior remnant reveals a definite lumen of 0.01–0.02 mm. in diameter. Subsequent to this period the short anterior remnant contains a definite lumen up to 0.03–0.04 mm. diameter, and in one case an expanded portion reached a diameter of 0.11 mm. The structure, therefore, shows definite growth in its anterior portion in normal males subsequent to day 50.

Table 1 indicates that 4 of the 13 males show evidence of androgenic stimulation of the Müllerian system. The greatest response occurs in animal 65A, sacrificed on day 39. The duct in normal males of this age is a solid nonluminate cord, whereas in this treated specimen a lumen was present which in one place was 0.09 mm. in diameter. Other males show definite, though less, stimulation than the male just cited. Figure 9 (Pl. V) (31-day male 64A, treated days 10–30) reveals the presence of a Müllerian duct with a small lumen that has been maintained over approximately its upper one-half. Since in normal 31-day males the duct is reduced to a solid nonluminate cord in its anterior portion, this case must be rated a response, even though of minimal proportions.

It is to be emphasized that androgens, though inducing some growth in size and the development of a lumen in Müllerian ducts in males, have failed to cause the ducts to grow in length or to prevent atrophy at their caudal ends. The youngest androgen-treated male (96A), sacrificed on day 16, reveals the Müllerian system to be solid degenerate cords extending approximately one-half the distance from the epididymis to the urogenital sinus. Thus, despite daily treatments from its first appearance, the usual postero-anterior retrogression has occurred, and a lumen was not established.

3. Testis responses: The testis responses to androgenic treatments have been relatively unimpressive; histologically, no outstanding or definite modification has been noted in these experiments at any age. In two cases attempts have been made to compare testis size in very young treated males with normal ones. Through utilization of the cutout-paper weight methods animal 47A, receiving testosterone ointment from day 3 to day 24, showed a total testis mass 30 per cent greater than that from a 24-day normal animal. Animal 64A, sacrificed on day 31, following injections of testosterone propionate on alternate days from day 10 to day 30, indicated a testis mass exceeding the normal control by as much as 81 per cent. These two estimates suggest increase in size of the testis at this age. At a later age, when the testis could be weighed immediately on removal and before fixation, this tendency did not continue. Thus, of two littermate males, sacrificed on day 83, the actual fresh weight of a single testis from an untreated male was 21 mg., whereas that of the treated was 24 mg. (treatment: 0.5 mg. *t*-propionate daily for 70–82 days). A second comparison between nonlittermates, but of the same body length and comparable weight, sacrificed on day 100, showed the normal testis weight to be 72 mg. and the treated testis 70 mg. (treatment 0.5 mg. *t*-propionate daily for 70–99 days). Two other animals sacrificed at an age estimated to be 1 year, and of comparable size, gave a weight of the two normal testes as 2.50 gm. and of the two treated testes as 2.40 gm. (treatment 5.0 mg. *t*-propionate daily for 33 days prior to sacrifice). Since histological study of all these cases revealed no characteristic or definite morphological differences in the treated organs, one must conclude that at these ages testosterone

and *t*-propionate in the dosages employed failed to cause perceptible modifications in the testis. It must be emphasized that the prostate gland, Cowper's gland, and phallus in these same animals revealed marked stimulation. The weight of the entire male reproductive tracts of the animals sacrificed on day 83, when compared, revealed that the reproductive system of the treated one was four times as heavy as that of the untreated normal; similarly, in the 100-day specimens the treated whole system was seven times the weight of the normal.

4. Prostate gland: The development of the prostate gland has been stimulated in all males treated with androgens and is perhaps second only to external genitalia in revealing responses to both early treatment and low dosage.

Chase (1939) has recently studied the normal prostate gland in the opossum, its origin and development, its final anatomy, and its response in adults to hormone manipulation. With the probable exception of such minor points as the state of development relative to embryonic age (my material being more abundant and more accurately timed) my observations offer confirmation of Mrs. Chase's account. Evidences of the prostate anlage as buds from the epithelium of the urogenital sinus appear on day 15. By day 32 (Pl. V, Fig. 10) prostate buds project from the entire surface of the anterior portion of the urogenital sinus epithelium, and by the 40th day the single bud outgrowths show beginning branchings permeating the surrounding mesenchyme. By the 100th day these branching outgrowths have laid the foundation for the final type of prostate gland possessed by this animal, but frequently canalization of the outgrowths has not occurred (see Pl. V, Fig. 14). In one normal male of 8 months the central ends of the outgrowths had become slightly luminate, with the outer two-thirds of the glands still solid epithelial cords; whereas in a second 8-month animal lumina extended to the peripheral terminations of the outgrowths, but the secretory cells were as yet quite below a functional level. In 3 males examined at an estimated age of 1 year the structure was assuming adult secretory characteristics.

As a means of representing responses of the prostate gland to androgenic treatments, Table 1 carries a list of measurements of the entire cross-section of the urogenital sinus and prostate outgrowths in comparison with similar aged normal animals shown in parentheses; this includes not alone the prostate outgrowth but the cavity of the urogenital sinus as well. Included in the table also is a subjective rating of prostate-gland response which is an attempt to portray comparative responses of the prostate outgrowths irrespective of growth in diameter of the entire urogenital sinus or its lumen. One can easily note that relative response of prostate-bud stimulation is not always parallel to increase in cross-section measurements. Animal 30A, despite a slight increase in total cross-section of urogenital sinus, reveals a prostate showing a high grade of precocious development. The response of the prostate of the 30-day-old treated male 101A (treated days 3–39) can be seen by comparing Figure 11 (Pl. V) with a normal 32-day-old male (Pl. V, Fig. 10); the comparison is direct by virtue of the same magnification.

Animal 64A, treated from day 10 to day 30 and sacrificed on day 31, was compared with an untreated 31-day male by means of weighing paper cutouts of the prostate tissue projecting from the surface of the urogenital sinus epithelium outlined at a magnification of 66×. Such weight comparisons of the prostate tissue in the two animals revealed that the prostate of the treated male was ten times that of the normal untreated prostate.

The variations in response in the treated series is naturally great, since sacrifice occurred at different ages and after different methods of application or different lengths of treatment. Designations of comparative response noted in the table is an attempt to portray relative responses, and in each case judgment was made in comparisons between similar-aged treated and normal young.

5. Phallus: Phallic stimulation has occurred at all ages and with all dosages of androgens employed; in general, responses have been greater with advancing age. The phallus of untreated males grows slowly, since on day 16 measurements were 1.5×1.0 mm. and on day 44, 1.8×1.5 mm.; no untreated phallus up to 50 days of age gave greater measurements. All measurements of this structure were made from Bouin-preserved young in 80 per cent alcohol and hence are readily comparable. In comparison with these data from untreated males, it will be seen from the table that all males listed show a larger organ despite sacrifice of some treated specimens by day 16 subsequent to not more than 10 days of treatment. Enormous responses follow treatments begun on day 70 or later. In several cases the organ has assumed entirely adult proportions and morphology with the characteristic bifurcated glans.

In summary, treatment of male pouch young with androgenic substances results in strong stimulation of the prostate gland, Cowper's gland, and the phallus; mild stimulation of the Müllerian ducts; less evident stimulation of the Wolffian ducts; and little or no effects on the testes.

B. *Effects on females.*—The responses of the developing reproductive system of female pouch young to androgenic treatment have been studied in 23 serially sectioned, treated young, ranging in age at autopsy from day 16 to day 147. From this group 16 females have been chosen for tabulation of records of observations (Table 1) in such manner that the 11 youngest females could serve in comparison with littermate males simultaneously removed from the nipple for preservation following identical treatment; the older 5 females represent reactions at later ages, as well as results following discontinuance of treatment. The 7 females receiving no mention revealed entirely similar responses to those listed in the table; included among those listed are representatives from all types of androgenic treatment. In general, sensitivity to modification in development has followed in a descending order from phallus, prostate, Müllerian duct, Cowper's gland, Wolffian duct, to ovary (no appreciable response). Since Cowper's gland and ovarian responses will receive consideration elsewhere, they will be given but the briefest mention here.

1. Wolffian ducts: The youngest female (96B), sacrificed after androgenic treatment, was preserved on day 16, following daily treatments since day 3. Detectable modifications in Wolffian ducts were not apparent. Treatments of female 63B from day 10 to day 21 resulted in visible modifications on day 22. Normal 24-day female Wolffian ducts are decidedly degenerate. Connections with the urogenital sinus are represented by a solid cord of cells in the genital cord; more anteriorly the duct in the degenerate mesonephros, and slightly posterior, exhibits a barely discernible lumen. The ducts in treated female 63B on day 22 were provided with lumina from anterior levels to the region of the lateral vaginal canal; between the latter and the urogenital sinus it was a solid cord; anteriorly, the lumen had a diameter of 0.01 mm., but more posteriorly this attained a diameter of 0.04 mm., which compares favorably with the duct in a normal male of this age. The ducts in female 47B showed somewhat greater stimulation but also failed to show openings into the sinus.

Female 101B, treated with androgens between days 3 and 30, instead of showing the normal female Wolffian duct composed of a solid cord of cells and falling considerably short of the urogenital sinus, contained luminate Wolffian ducts from the efferent tubule area, anteriorly, to the level of the lateral vaginal canals; luminal diameters of 0.03–0.04 mm. compare well with normal males of this age but in one area the experimental ducts reached a diameter of 0.11 mm. and failed to show entry into the sinus cavity. In treated female 64B, sacrificed on day 31, the Wolffian-duct outline can be seen faintly along the greatly enlarged Müllerian system (Pl. IV, Fig. 6). In sections (see Pl. V, Fig. 8) the Wolffian duct is seen as a luminate tube from the epoöphoron region to the level of the lateral vaginal canals; the luminal diameter is again approximately 0.04 mm. over its larger portion, but in the walls of the lateral vaginal canal it attains a diameter of 0.26 mm.

Female 66, treated with androgens between days 10 and 31, received no further treatment up to sacrifice on day 62. Despite the post-treatment recovery period, Wolffian ducts were luminate throughout, with the usual exception of the portion from the posterior part of the lateral vaginal canals to the urogenital sinus; this portion was reduced to a solid cord. The lumen anteriorly had a diameter of 0.03 mm. but along the uterine level this increased to 0.15 mm. and became greatly expanded along the walls of the lateral vaginal canals (0.75 mm.).

Female 117 received continuous treatment with androgens from day 3 to day 93; and, whereas the epoöphoron tubules were well developed, with luminal diameters up to 0.06 mm., the duct proper was reduced to a lumen of 0.02 mm., along side the uterus; in the region of the lateral vaginal canals the duct is further reduced to a nonluminate cord that can be traced to contact with the urogenital sinus epithelium. Female 118 was sacrificed at the same age but had gone without treatment between days 45 and 95, yet the Wolffian ducts were quite similar to those of female 117; in the lower uterine level a short segment was expanded to a luminal diameter of 0.37 mm. Female 120 was a littermate and received identical treatment to female 117 up to day 95; it was sacrificed without further treatment on day 147. Wolffian duct preservation differed in no remarkable way from female 117.

Generally speaking, the responses of the Wolffian ducts in females that had received continuous treatments with androgens, from a period much earlier than the beginning of duct regression following loss of urinary function, have not been striking. The epoöphoron tubules, fairly conspicuous in normal females of the opossum, revealed rather consistent responses, characterized by healthy preservation and increase in size. The more posterior portions of the ducts have given quite varied responses in the different treated females; but in all cases the ducts have been recognizable, usually with a small lumen, as far posterior as the lower levels of the lateral vaginal canals. Frequently, localized portions have become quite expanded, and such areas usually contain cellular debris. Only in female 107 was the connection with the urogenital sinus approximately normal for untreated males. Whereas bilateral preservation is usual, the posterior portions are quite characteristically reduced to solid epithelial structures in which a visible lumen is absent. Frequently along the lateral vaginal canals the ducts may be thrown into folds, as if a growth in length was stimulated. Androgens have preserved the ducts in females, therefore, but the preservation in most cases is atypical for normal males.

2. Müllerian duct: The stimulation of the Müllerian duct in female opossum pouch young from androgenic treatment has been a striking feature of this study, especially when comparisons are made with reactions of the same structure in treated males. All androgen-treated females have shown Müllerian-system stimulation, irrespective of

dosage, method of treatment, or age at sacrifice; it is true, however, that with advancing age or maturity of tissues the responses have been more pronounced.

The youngest female preserved after treatment (96B) was sacrificed on day 16 subsequent to daily application of testosterone ointment from day 3 to day 15. Normal females at this age have the Müllerian system represented by cords of cells continuous from the ostium to contact with the urogenital sinus; in occasional areas along the cord a tiny lumen is apparent, but for most of the distance the cord has not acquired a lumen. In this treated female a lumen is present throughout, though it is greater at the genital-cord level, where the diameter is 0.03 mm.; the lumen, however, is not traceable into the cavity of the urogenital sinus, as holds for all specimens under 100 days of age. The androgenic stimulation in this case, though definite, is minimal, as can be seen in the 22-day treated female.

Female 63B, sacrificed on day 22 subsequent to injections of *t*-propionate from day 10 to day 21, has been compared with a normal female of 24 days in order to attempt to portray the responses from androgenic treatment. In the normal 24-day female the various differentiated regions of the Müllerian duct have just become evident. The tube is essentially straight, with a lumen barely evident at the oviducal level but becoming more prominent at the uterine level (diameter 0.01 mm.), narrowing for the cervical region and expanding (diameter 0.01 mm.) for the lateral vaginal canal. When a comparison is made of this normal 24-day with the treated 22-day female, it is found that the normal oviduct lumen is barely perceptible, whereas treated oviducts are tortuous and show a luminal diameter of 0.03 mm. The normal uterus, with a total cross-section of 0.18 mm. and a lumen diameter of 0.01 mm., becomes in the treated female 0.87 mm. in total cross-section with a luminal diameter of 0.52 mm. Lateral vaginal canals of the normal have an entire cross-section of 0.18 mm. and a lumen of 0.01, whereas the treated lateral vaginal canals are 1.35 mm. in total cross-section and show a lumen of 1.10 mm Thus, the lumen of the treated uterus has a diameter approximately fifty times as great as the normal, and a lateral vaginal canal lumen one hundred times as great. The uterine epithelium of the treated female, while active, has not produced definite glandular outgrowths.

Treated female 64B, sacrificed on day 31 following *t*-propionate injections from day 10 to day 30, can be seen dissected to show the reproductive system in comparison with that of a normal 31-day female (Pl. IV, Figs. 5 and 6). From serial sections of these two female embryos one finds that the normal female uterus is 0.22 mm. in total cross-section and the lumen 0.07 mm. in diameter, whereas the uterus from the treated female is 1.65 mm. in total cross-section with a lumen 1.12 mm. Similar measurements for the lateral vaginal canals reveal the normal total cross-section to be 0.20 mm. and the lumen 0.06 mm., whereas in the treated female the total cross-section is 3.30 mm. and the lumen 3.00 mm. Rough comparison can be expressed by stating that the total uterine cross-section in the treated female is 8× normal and the lumen 16× normal, or the total lateral vaginal canal cross-section is 13× normal and the lumen 50× normal. In point of differentiation the treated uterus contains numerous well-formed glandular outgrowths which do not occur until several months of age in normal females. In female 64B the epithelium of the lateral vaginal canals has not advanced in differentiation to an extent comparable to uterine differentiation. Uteri of these two females, photographed at the same magnification, are shown in Figures 7 and 8 (Pl. V).

Such tremendous increases in the size of Müllerian ducts in the above case, and in other treated females not described in detail, are frequently characterized by distention of the tube; it becomes evident that tissue growth has occurred, but it is similarly evident from microscopical study that differentiation has likewise been stimulated. Uterine glandular differentiation has advanced remarkably, and the mitotic index of many tissues is very high. To cite two examples, treated female 42 (not listed in the table) received testosterone-ointment treatment between days 16 and 50, with sacrifice on day 51. Caliper measurements of the lateral vaginal canal prior to fixation has been recorded as 6 mm. × 3 mm. When one examines the uterine glands in this female, the evident growth and differentiation of the uterus is revealed by numerous glands with an extremely high mitotic index; quite consistently, cross-sections of these glands reveal approximately each 8th to 10th nucleus in mitotic division—truly an extreme stimulation of mitosis in these nuclei of uterine glands. Female 47B, treated with testosterone ointment from day 3 to day 24 reveals moderate lateral vaginal canal stimulation (diameter 0.30 mm.; compare with treated female 63B, diameter 1.05 mm.); yet the mesenchyme cells (future muscle) surrounding the epithelium are extremely active. It is entirely consistent to see, within a single microscopic field (4-mm. objective), from 15 to 25 mesenchymal nuclei in mitotic division.

Tremendous responses to androgenic treatment characterize Müllerian reactions in all females, but older females differ from younger ones principally in greater increases in thickness of Müllerian duct walls and relative reduction in the lumen. This is due, to some extent, to the enormous glandular proliferations, more especially in the walls of the uterus than in the lateral vaginal canals. And, when once developed, the glands show but little involution after cessation of treatment, as may be seen in the case of the three older specimens listed in Table 1.

Females 117 and 118 were preserved on day 95, the former having received daily treatments from the third day until sacrifice, the latter having treatments discontinued on day 44, hence without treatment for the last 51 days. Figure 21 (Pl. VI) is a cross-section of the uterus of treated female 117, sacrificed on day 95, which should be compared with the uterus of a normal 100 day female (Pl. VI, Fig. 22). Since the figures are photomicrographs at the same magnification, comparison readily demonstrates the character of the response. The uterine wall of treated females is greatly thickened, glandular differentiation has been profuse, and the lumen of the tube is considerably reduced. Glandular formations are not indicated in the normal uterus at this age. The uterus of female 118 compares quite favorably with that of female 117, despite the fact that the former had received no treatment between days 45 and 95. Some glandular involution is apparent, but this is not great. In a similar manner the uterus of female 120 exhibits well-developed uterine glands despite the absence of treatment from day 96 to sacrifice on day 147.

3. Ovarian responses: The ovarian responses to androgenic substances will form the material for a separate account, and it will be indicated here only that marked modification in development has not been evident. By the utilization of projected sections on standard paper and of the weight of these sections, there is some indication that the mass of the ovary has been increased to a slight extent by androgenic treatments.

Histologically the opossum ovary presents many interesting problems, which will receive attention elsewhere, and it will be stated only in general that after any androgenic treatment the ovarian structure is not markedly modified. Its histological character appears thus far to have undergone some slight changes, but in any treated female ovarian activity is evident in its various stages of organization. Older stages show well-formed normal appearing follicles, and the germinal epithelium at all stages of preservation appears active.

4. Prostate development: In females prostate development has not only been induced by treatment with androgens, but the degree of development and differentiation has also been surprising; normal females fail to develop the anlage of this structure at any stage. Such an animal as the rat may reveal prostatic homologues in the female in early development, only to have them regress and disappear entirely, or remain as rudimentary "paraurethral glands of Skene."

It is to be recalled that the prostate anlage in normal males appears on day 15. Despite its total absence in normal females, a treated female (96B) sacrificed on day 16, subsequent to 13 daily treatments with free testosterone ointment, showed prostate development of the characteristic male type, as well-formed buds growing out from the epithelium of the urogenital sinus; the extent of these outgrowths in this treated female is greater than in the normal male of similar age. Induction of a prostate by androgenic treatment, as well as precocious development, is here evident.

Prostatic responses in treated females are indicated in Table 1 by actual measurements of cross-sections of the urogenital sinus and prostatic outgrowths and, more adequately, by subjective ratings. Measurements, though reflecting increases in the total structure, do not differentiate between enlargements of the urogenital sinus cavity and the extent of actual prostatic outgrowths. Thus, in female 101B the diameter of the urogenital sinus and prostate is but slightly greater than the urogenital sinus diameter in a normal female of similar age; yet the response of the glandular outgrowths has been rated as "great stimulation."

A section of the urogenital sinus showing prostatic outgrowths in this treated female sacrificed on day 30 is shown in Figure 12 (Pl. V), photographed at the same magnification as is that of the normal 32-day male in Figure 10 (Pl. V). The much greater development in the treated female than in the normal male is evident; and if the comparison is made with the normal female, which shows no evidence of prostate outgrowths, the rating of "great stimulation" appears justified. The treated male prostate 101A, receiving identical treatment to its sister, shown in Figure 11 (Pl. V), although greater in amount, has received a lower subjective rating because the response has occurred in a male which would otherwise have had a prostate equivalent in development to Figure 10 (Pl. V). The grade of differentiation in the treated male prostate (Pl. V, Fig. 11) is approximately equivalent to a normal male of perhaps four months; that in the treated female prostate (Pl. V, Fig. 12) is equivalent approximately to 80–90 days of normal male development. It may appear that higher ratings are given to female prostate than to male prostate responses, but in each case the treated specimen is compared with a normal same-sexed individual and not with each other; female responses may, in a few cases, represent less total prostate tissue than in a similar aged male and yet receive a higher rating in relative response. In the 31-day treated female the prostate gland was compared by the paper-cutout method with that of a treated brother (64A) and a normal 31-day old male. In serial (10 μ) sections the total number of prostate sections in the normal male was 57; in the treated female, 131; and in the treated male, 153. By projecting prostate outgrowths at a magnification of 66X on standard paper and weighing the cutouts, it is revealed that the mass of prostate epithelium in the treated female is six times that of the normal male, while that of the treated brother is ten times the normal male.

Induced prostate development in treated females is not followed by immediate regression on cessation of treatment. Thus, females 118 and 120 each possess prostate glands rated "extreme stimulation" despite the absence of treatment during the last 50 days of life. Figure 15 (Pl. V) is a section of the urogenital sinus and prostate outgrowths from female 118 treated between days 3 and 44 and remaining without further treatment until sacrifice on day 95. When compared with Figure 14 (Pl. V), a section of the same region from a 100-day normal male, it is evident that the female prostate shows markedly greater differentiation despite the lack of treatment for the last 50 days of life.

5. Phallus: Phallic development in normal females is quite indistinguishable from that in normal males up to an age of approximately 30 days; in each case relatively slow growth occurs, and in normal animals measurements of the structure do not exceed 1.5×1.0 mm. On day 44 measurements of the phallus of the male were 1.8×1.5 mm. and for the female 1.5×1.0 mm. Thus, near the age of 50 days, phallic growth in the two sexes diverge, with the male showing more rapid subsequent growth.

The female phallus, like the male, exhibits marked growth responses to androgenic treatment. Attention to Table 1 reveals that the phallus of all treated females has responded by an increase in size over normal; the weaker response in females 63B and 38A has still produced organs larger than in normal females of like age. Also, the table shows that the phallus of littermates receiving identical treatment is larger in the male than in the female in 10 of the 11 pairs; female 101B had a phallus somewhat larger than the brother 101A. Treatment of females with androgens for longer periods, as in female 117, sacrificed on day 95, reveals the capacity of the female phallus to differentiate in the male direction. In this case the female phallus was considerably greater than in normal males of the age, and differentiation had rendered it a typical male phallus with bifurcate glans characteristic of the mature male organ. This clitoris, therefore, has undergone development entirely similar to that of the penis in males, and it compared favorably in size with that of a young mature male.

Since Burns (1939*d*, 1939*e*) has given considerable attention to phallic modification in opossum pouch young and since my materials confirm his main points, further details may be omitted. It is to be emphasized, however, that the phallus of the male shows somewhat greater growth responses than does that of the female. Furthermore, phallic modification appears to be one of the first to follow androgenic treatment, both in point of time and in point of response to low hormone dosage; the phallus and the prostate gland register the first of changes that follow this type of treatment.

C. *Comparative reactions of males and females to androgens.*—In the preceding pages attention has been directed largely to the effects of androgens on individual structures of males or females as these effects become evident from comparisons between the treated animal and the untreated normal one of the same sex and age. There appears to be some advantage in emphasizing the responses of the same structures to the same treatment in the two sexes. For this purpose it is of particular advantage to compare littermates sacrificed at the same time; this insures, as nearly as possible, a differential of sex only, since all members in a given pouch were treated exactly alike during the same period of development and with the same substance. For this purpose three pairs of pouch young will be compared at ages of 24-day, 30-day, and 39-day sacrifice, which well covers the period of heterosexual differentiation of the Wolffian and Müllerian systems. Entirely similar relations hold for all other pairs, as well as for isolated individuals from a pouch, and in many cases even greater differences are shown than in these three pairs; one chief reason for the selection is the early age at which treatments were begun.

Twenty-four-day-pair: Pouch young 47A and 47B were sacrificed on day 24 subsequent to androgenic treatment daily from day 3 until the day of sacrifice. Wolffian-duct response in the male was essentially absent, but in the female (41B) it received a rating of "moderate stimulation." In a normal female the structure would have been reduced to a condition exhibiting the barest cavity for a lumen, and the solid cord posteriorly would have made an attachment with the urogenital-sinus epithelium. This treated female possesses a Wolffian duct with a lumen of 0.06 mm.; hence, in comparison with a normal female, it was stimulated. In point of relative comparison there is little difference in the duct at this age between a normal male and this treated male or female. Despite the evident stimulation in the female, therefore, the duct has been retained in essentially its typical state of development in normal males: in other words, regression of the structure in the treated female has been prevented.

Müllerian duct responses in the two sexes is materially different. Tabulated ratings for this pair show Müllerian ducts "slightly stimulated" in the male and "moderately stimulated" in the female, but the extreme difference between the end-products in the two sexes is not thus adequately represented. In normal males at 24 days Mullerian ducts are reduced to solid cords ending approximately one-half their length toward the urogenital sinus. Because of the development at anterior levels of a definite, though very small, lumen, slight stimulation over the condition in normal males is registered. As a general estimate, not more of the original duct persists than in the normal male, but the part which is present exhibits somewhat better development. In the normal female of 24 days the differentiated portions of the duct system would be barely indicated, and the lumen would have a gross diameter of 0.01 mm. The Mullerian system of the treated female, in exhibiting the definite formation of its various portions having lumina greatly in excess of the normal (oviduct, 0.03 mm.; uterus, 0.11 mm.; lateral vaginal canal, 0.30 mm.), has received a rating of "moderate stimulation." It must thus be evident that the Mullerian ducts of females respond to the same treatment with androgenic substances with an intensity entirely different than in males.

Thirty-day-pair: Comparisons of the duct responses to the same androgenic treatment in males and females sacrificed on day 30 may be made between pouch young 101A and 101B. These received daily treatments with androgenic material from day 3 until day 29.

The Wolffian ducts of the treated male (101A) were rated "slight stimulation" because the diameter of the ducts in the treated male was, in some areas, three times that of the normal control (0.09 and 0.03 mm). Luminal connections with the urogenital sinus had not been retained, and the usual solid cord termination was present. In the female (101B) "moderate stimulation" of the Wolffian duct was recorded. This is on account of the fact that in normal females of this age Wolffian ducts are reduced to solid cords of cells not reaching the urogenital sinus, whereas in this treated female the structure remained as a continuous tube with a luminal diameter of 0.03 to 0.11 mm. It did not continue posteriorly farther than in the normal female, but the portion remaining had been converted into a definite luminate tube. Thus, despite androgenic treatments since day 3, the full length of the duct was not retained.

Müllerian ducts exhibit an immense difference in the two sexes receiving the same hormonal treatment. In normal males of this age the Müllerian system would have been reduced to a solid cord extending approximately one-half the distance to the urogenital sinus. In the treated male it was present over approximately the same distance, but anteriorly it possessed a lumen of 0.01 mm., hence the rating "slight stimulation." In normal females the duct would have shown a maximal luminal diameter of 0.03 mm., and this would have been at the lateral-vaginal-canal level. The treated female exhibited a luminal diameter of this portion of 1.42 mm., or approximately forty-five times normal, and a uterine diameter of from seven to ten times normal; the uterus contained well-developed glandular formations; hence the rating of "great stimulation" was recorded. But consideration of the Müllerian response in the male, as compared with the female, emphasizes that the comparison is between a tube one-half its length, having a lumen of 0.01 mm., and a well-differentiated complete female system having a maximum lumen of more than one hundred times the male. It is to be emphasized at this point, therefore, that Wolffian ducts, though responding modestly, are in the end approximately equal in the two sexes, whereas Müllerian duct responses in the two sexes show tremendous discrepancies despite treatments with androgens begun at an early age.

Thirty-nine-day-pair: Pouch young 65A and 65B are the oldest heterosexual pair of littermates to be sacrificed on the same day from the same pouch and therefore having had identical treatment. Many older males and females from different pouches are available, but the advantage in utilizing littermates for comparative duct reactions will be evident.

Wolffian duct response in 65A was rated "slight stimulation" because the duct lumen was approximately twice that of normal male controls (0.06 mm. and 0.03 mm.). No other evident signs of stimulation of the duct were present with the exception that efferent tubules were apparently stimulated to some extent. In the female 65B "slight stimulation" was registered because in normal control animals this structure would have been a severely reduced solid cord of cells ending anterior to the genital cord. In the treated female it existed as a solid cord of cells still connected with the urogenital sinus, and throughout its course showed a lumen of 0.03-.06 mm.; a distended portion opposite the lateral vaginal canals possessed a luminal diameter of 0.37 mm.

Müllerian ducts again exhibit tremendous discrepancies in their responses in the two sexes. In the treated male the structure has been retained in approximately one-half its original length and anteriorly exhibits a lumen of 0.03 mm. diameter. The stimulation is therefore evident. In the normal female of this age the system would have possessed a maximal luminal diameter of probably 0.20 mm. for the uterus and 0.30 mm. for the lateral-vaginal-canal portion. This treated female had a uterus with well-developed glands and a lumen of 1.5 mm. and a lateral-vaginal-canal portion of 1.6 mm.

This tremendous difference in the Müllerian system of a male and female treated with androgenic substances since the 10th day, at which time Müllerian ducts in the two sexes are essentially alike and have just reached the urogenital-sinus area, clearly shows that this structure in the two sexes exhibits differential responses; the male Müllerian ducts and those of the female do not have the same capacities to respond to this hormonal treatment.

In a résumé of the effects of androgens on the early sexual differential of pouch-young opossums it may be briefly recalled that (*a*) the gonads of either males or females are not markedly affected; (*b*) Wolffian ducts of both sexes show only minor responses to androgenic treatment, and the structures are maintained in part or fairly complete in females; (*c*) Müllerian ducts respond in each sex but in a tremendously different manner; (*d*) prostate glands are markedly stimulated in both sexes, but induction in females occurs before the great growth responses can occur; (*e*) Cowper's gland in both sexes responds markedly; (*f*) the phallus is greatly stimulated in each sex, slightly more in males, and that of females is modified to the typical male type.

V. EFFECT OF ESTROGENIC TREATMENT

Estrogenic substances have been found to be decidedly toxic for pouch-young opossums. Such treatments have involved the subcutaneous injections of theelin in oil, injections of estradiol (progynon-B) in oil, estradiol in alcoholic solution applied to the skin surface, and estradiol in ointment form. Earlier treatments were largely fatal to embryos, probably because of too high dosage.

The majority of the pouch young have been treated by the application of a commercial face cream to the surface of the skin by means of a small brush. The face cream was purchased over the counter of a leading Chicago department store and was recommended to women as a preventive for facial wrinkles and a general skin beautifier. It contained 0.625 mg. of estradiol per ounce of cream, and its estrogenic properties and skin penetrability were rather thoroughly tested on adult rats and guinea pigs (Moore, Lamar, and Beck, 1938). Its capacity to produce, in very small quantities, cornified vaginal smears in spayed rats, uterine growth after ovariectomy, mammary-gland growth in male guinea pigs, and testicular injury in normal male rats are entirely in keeping with similar phenomena obtained through injections of estradiol in oil.

Ointment application is very difficult to quantitate, because of the fact that similar thin body coverings will require progressively larger amounts of the cream as the animals grow, and also because of the fact that pouch young are not only in contact with each other but also in contact with the mother's pouch. One is entirely unable to state approximate amounts of the hormone absorbed, but the amounts applied daily would be of the approximate order of 1 γ daily for younger stages and not over 6 γ for the older and larger pouch young.

With this type of treatment there was still a large loss of pouch young. Treatments of 5-day-old pouch young were often followed by a daily loss of 1–4 young from the pouch, and all members of a pouch may be lost before treatments have been given for 5 days. Approximately 10 litters have been started on treatment, and a typical example is the final preservation of 4 pouch young from a pouch in which treatments were begun on 12. In a few cases young were recovered from the cage before death occurred, and all dead young recovered have been preserved; some of these were fairly satisfactory for study. Consideration of effects will be restricted to young which were removed from the nipple at desired periods and to a few discovered while still alive but which had become detached from the nipple. Owing to the delicacy of younger individuals, 1 or 2 hours out of the pouch is fatal. All pouches were examined at 24-hour intervals during the course of treatments, and in many cases they were examined more frequently; despite this fact, many dead ones were unfit for study. The group to be considered here consists of 8 males and 11 females.

A. *Males.*—In Table 2 are to be found essential data on 8 male opossum pouch young receiving estrogenic treatment; these data include ages at beginning and termination of treatment, as well as brief notations representing responses of the reproductive system. Four males of the group represent treatments started at ages prior to completion of backward growth of Müllerian ducts toward the urogenital sinus, 2 started at ages subsequent to such contact, and 2 at periods approaching the time of detachment from nipples.

Ser. 97: The youngest male preserved, Ser. 97, as well as the one receiving the shortest treatment, received the first application of estrogenic ointment on day 6, when Müllerian ducts were represented by solid cords of cells extending approximately to the caudal end of the mesonephroi: it was preserved on day 12, 1 day after the last of 6 treatments, at an age when Müllerian ducts would have approached contact with urogenital-sinus epithelium. The treatments were apparently without demonstrable effects. Wolffian ducts were normal in caliber, and Müllerian ducts were represented by cords of cells just approaching the urogenital-sinus epithelium; no growth stimulus was apparent in these ducts.

TABLE 2

EFFECTS OF ESTROGENS ON OPOSSUM POUCH YOUNG*

SER. NO	AGE AT DEATH (DAYS)	TREATMENT (DAYS)	WOLFFIAN DUCT	MÜLLERIAN DUCT (MM.)				PHALLUS
				Oviduct	Uterus	Cervix	Lateral Vaginal Canal	
	Males							
97. .	12	6–11	0 06 (0 04)	Cord	Cord			1.5×1 0
49A	17	5–16	0 22 (0 06)	. . ..	0 15 (0 00)			2 0×2 0
59 . . .	17	10–16	0 07 (0 06)	. . . .	0 15 (0 00)			1 7×1 0
55A	23	5–22	0 60 (0 04)	. . . .	0.22 (0 00)			1 2×1.5
109. .	30	6–29	0 15 (0 03)	. . .	0 22 (0 00)		.	3 5×3 0
39A	31	17–30	0 06 (0 03)	. . . .	0 07 (0 00)			1 5×1 3
93 .	83	70–82	0 07 (0 03)	. . . . .	0 33 (0.03)			. .
95. .	88	70–87	0 07 (0 04)	. . . .	0 52 (0 03)			. . . .
	Females							
49B. .	17	5–16	0 90 (0 06)	0 01 (0 00)	0 30 (0 00)	. . .		2 0×2 0
60. . .	19	10–18	0 33 (0 06)	0 01 (0 00)	0.18 (0.00)			2 0×1 2
100. .	22	6–21	0.60 (0 00)	0 01 (0.00)	0 45 (0.01)	0 07 (0 00)	1 35 (0 00)	4 1×3 0
55B	23	5–22	0 45 (0 00)	0 02 (0 00)	0 33 (0.01)	0 07 (0 00)	0 15 (0 01)	1 6×1.6
108. .	30	6–29	2 15 (0 00)	0 03 (0 01)	2 85 (0 02)	0 07	2 15 (0 03)	4 0×2 0
106. .	35	6–34	1 30 (0 00)	0 04 (0 03)	1.80 (0 07)	0 09 (0 03)	1 80 (0.11)	6 0×5 0
114. .	39	6–38	0.82 (0 00)	0 03 (0 03)	3 00 (0.30)	0 07 (0 07)	0 70 (0.37)	4 0×4.0
110. . .	43	6–42	2.10 (0.00)	0 03 (0 03)	2.70 (0 30)	0 07 (0 07)	1 70 (0 37)	4 0×3 5
111. .	46	6–45	1 50 (0 00)	0 03 (0 03)	4 50 (0 30)	0 07	1 20 (0 37)	5.0×2 0
115. .	46	6–45	1 80 (0 00)	0 03	2.85 (0 30)	0 07	1 45 (0 37)	4 5×4 0
119 . . .	79	{6–45 46–79 no tr}	0 75	0 04	1 80 (0.65)	0 07	1 50 (0 75)	4.1×1 9

* Greatest diameters of lumina of ducts in millimeters; ocular micrometer measurements from fixed preparations. Figures in parentheses show diameter of lumen in normal of same age and sex. Phallus, millimeters.

Ser. 49A: This 17-day-old treated male shows modification. Wolffian ducts in 4 untreated males on the 16th day revealed patent ducts throughout their course with a luminal diameter not exceeding 0.06 mm.; and, in each of these young, connections of the lumen with the cavity of the urogenital sinus were still perceptible. In this male, treated with estogens by ointment, the Wolffian duct likewise entered the urogenital sinus, but it was sufficiently enlarged along its course to show a maximal luminal diameter of 0.22 mm. (over 3× normal). Müllerian ducts, which in normal males were reduced to solid cords and terminated anterior to the genital cord, were large luminate ducts. Anteriorly an ostium was not seen. The duct, in its course through the mesonephros region, contained a well-defined lumen which more posteriorly increased to a diameter of 0.15 mm. At the posterior end the ducts ended blindly considerably anterior to the urogenital sinus. This region itself exhibited a tremendous hyperplasia and a central sloughing of its innermost layers of cells.

A prostate gland, visible in all four 16-day-old control males, was not in evidence in this 17-day-old treated male.

Ser. 55A. Marked response to estrogenic treatment was given by a male sacrificed on day 23. At this age normal males show marked reduction in Wolffian ducts, subsequent to loss of mesonephros function; a Müllerian duct represented by a solid cord of cells terminating considerably anterior to the urogenital sinus; and a prostate gland well represented by outgrowths from the urogenital-sinus epithelium.

This treated male had Wolffian ducts showing patency throughout their course and containing considerable cellular debris in the lumen. The lumen of the duct was small anteriorly in the substance of the mesonephros (luminal diameter 0.07 mm.), but more posteriorly it was enlarged to a luminal diameter of 0.60 mm. (20× normal). The lumen was traceable into the uppermost part of the urogenital-sinus horn, but hyperplasia was so great that the actual luminal connection with the latter cavity is somewhat questionable.

Müllerian ducts terminated blindly at both ends; an ostium was not present; and the segment representing the oviducal level was composed of isolated, disconnected short luminate pieces. In the caudal portion of the degenerating mesonephros the duct became continuous, and posteriorly it terminated quite anterior to the genital cord and attained a luminal diameter of 0.22 mm. Figure 18 (Pl. VI) is a section through this estrogen-treated male, which should be compared with the androgen-treated male 64A (Pl. V, Fig. 9) for relative duct responses.

The prostate gland was not visible. The entire urogenital sinus was a very large solid mass of cells peripherally, and centrally numerous layers of dying cells were being sloughed into the lumen; the hyperplasia was pronounced. This type of response of the prostate region may be seen in Figure 13 (Pl. V), which is a section of estrogen-treated male 39A, sacrificed on day 31.

Ser. 93–95: Representing treatment on older males, series 93–95 are particularly interesting because of effects on the prostate gland. Daily subcutaneous injections of 20 I.U. progynon-B were begun on day 70. Sacrifice of series 93, along with an untreated male control, occurred on day 83, and it was anticipated that treatment would continue on series 95 until day 100; on day 87, however, this male was perceptibly edematous, and the reproductive tract was removed shortly after death on day 88. The following brief remarks refer to series 93, sacrificed on day 83.

The entire reproductive tract was preserved in an extended position in Bouin's fluid; and, after transfer to 80 per cent alcohol, the weight of the entire tract (minus bladder, and one testis otherwise preserved) was 0.3842 gm., while that of the normal control was

0.1148 gm. Thus, despite a total body weight for the untreated male (72 gm.) greater than that of the estradiol-treated male (56 gm.), the reproductive tract of the latter weighed more than 3× that of the normal. The prostate-gland area of the treated male was much larger in gross size, and at comparable levels the diameter of an entire cross-section was approximately three times that of the normal control. Histological sections of this stimulated prostatic region show clearly that, whereas estrogens impart a tremendous growth stimulus to this area, the reaction is not one of prostate differentiation but a tremendous hyperplasia and metaplasia of the epithelial elements. Figure 16 (Pl. V), illustrating the reaction, should be compared with Figure 14 (Pl. V), a section of a 100-day normal male prostate and urogenital sinus. It is clear that a marked hyperplasia, involving the branching cords of prostate epithelium, has been induced and that the entire extent of the prostate cords is involved. The reaction is similar to responses of the urogenital-sinus epithelium to estrogenic treatment of the 31-day male shown in Figure 13 (Pl. V). This typical hyperplasia and metaplasia has followed estrogenic treatments in males, as well as in females, and no sexual difference in the response of the urogenital sinus has followed such treatment except in the case of males, in which treatment was begun after prostate development had advanced. In early stages, therefore, all prostate development in males is suppressed, whereas in later stages the outgrowths are markedly modified.

Testis development, or testis weight, in comparison with that of the normal male, was not modified during the course of 13 days of treatment; weight and histological character reveal no perceptible change.

Wolffian ducts, represented by the vas deferens and epididymal tubules, showed an increase in luminal diameter which was approximately double that of the normal; and the remnants of Müllerian ducts, representing a blind persistent remnant along the course of the epididymis, showed great increase in size as indicated in the table.

In general, estrogenic treatments of developing males, especially prior to day 30, have resulted in stimulation of both Wolffian and Müllerian ducts, the intensity of stimulation for both ducts being lower than that for the same ducts in females. The responses of Müllerian ducts in males to estrogens might have been anticipated, but the character of response may be unexpected. The 6 males receiving treatment during the period of caudal growth of these ducts failed to demonstrate the capabilities of the hormone in promoting more rapid growth in this direction or of retaining the portion of the duct which already had developed; despite the treatments, the ducts regressed in a postero-anterior direction and ended blindly. The responses, therefore, are essentially restricted to an increase in size of those remnants that have been maintained. Anteriorly the ducts are not retained; ostium development shows no stimulation, but rather regression; and the oviducal portion is largely absent despite continuous estrogenic treatment. Stimulation is shown from the fact that, at ages when Müllerian ducts in normal males would be reduced to solid cords of cells, these ducts in treated males were relatively large luminate ducts lined with a good epithelium; but these growth-stimulating phenomena are not in a direction of normal morphological differentiation; glandular developments are not evident. Wolffian ducts, likewise, demonstrated some increase in size, but this becomes less evident with increasing age.

Effects of estrogenic substances on the urogenital sinus have been principally the induction of a distinct and pronounced hyperplasia, with proliferation of cells proceeding centrally and with sloughing of cornified-type layers into the lumen. The germinative layer is always on the outside; and, instead of the normal prostatic outgrowths in the form of cords of cells proceeding peripherally, all cell movement has been directed centrally—a type of polarity reversal, as far as the growth tendencies of the epithelium are concerned. Furthermore, prostatic outgrowths, well formed prior to the beginning of treatment, are themselves subject to hyperplasia and to metaplasia, with a conversion of the orderly processes in development into a more or less unorganized, riotous, development of masses of relatively undifferentiated cells.

B. *Females.*—In Table 2 is a list of 11 females, subjected to estrogenic treatment for different total periods and at different ages, that will receive consideration here. As a measure of response the diameter of the lumen of the ducts, as well as the different segments of the Müllerian ducts, are listed as determined from sections by an ocular micrometer; frequently, outside diameters are ill defined because of contiguous structures, but, even though more accurately determined, luminal diameters fail to show thickness of walls of the structures listed. Treatments on the majority of these females were begun on day 5 or 6, since at this period Müllerian ducts are in process of formation; they are growing caudally but will not yet have reached contact with the urogenital sinus.

Detailed consideration will be restricted to females 49B and 115, representing the youngest and oldest continuously treated individuals of the group.

Ser. 49B: This female received daily treatments with estradiol ointment from day 5 to day 16 and was removed from the nipple on day 17. On this day a normal female possesses a Wolffian duct, into which open the mesonephric tubules, and the duct possesses a well-defined lumen extending to the urogenital sinus, but the actual luminal entrance may be barely interrupted: its greatest lumen diameter is 0.05–0.06 mm. The Müllerian duct barely, if actually, contacts the urogenital sinus, and throughout its course from the ostium posteriorly there will be no regional differentiation; simple columnar epithelium will, for the most part, fail to reveal a lumen, but in places the lumen will be evident.

This treated female, in contrast to the normal, reveals a tremendously stimulated set of ducts. The Wolffian duct is complete from the efferent tubule area anteriorly to definite contact with the urogenital-sinus horn. Anteriorly the lumen is 0.11 mm., but caudally it gradually increases in diameter to 0.37 mm. in the uterine area; and just anterior to contact with the urogenital sinus the lumen reaches a diameter of 0.90 mm.—a diameter of more than fifteen times that of the Wolffian duct in a normal female of the same age. Considerable cellular debris is present in the duct, some of which may represent degenerating kidney material, some from cells possibly cast off from the Wolffian duct wall and from other cells that may have sloughed from the greatly stimulated urogenital sinus. Figure 19 (Pl. VI) is a cross-section through the ducts of the 23-day treated female, 55B, and may be compared with sections of a normal 31-day female (Pl. V, Fig. 7) or the androgen-treated male in Figure 9 (Pl. V). The urogenital-sinus epithelium has experienced a tremendous hyperplasia leading to large solid cellular masses and broken down, cornified, epithelium sloughing into its cavity (similar to Pl. V, Fig. 13).

The Müllerian duct, which in a similar-aged normal female would be solid, or barely revealing a lumen, is differentiated in the treated female into an oviducal portion (lumen 0.015 mm.) and a well-developed uterine region divided into internal partitions from foldings, suggesting beginning glandular formations; the maximal lumen diameter of 0.37 mm. represents an increase of approximately forty times that in the untreated normal female. The duct becomes narrower, and shortly caudal to a point where the luminal diameter is 0.06 mm. it ends blindly without clearly contacting the urogenital-sinus epithelium; no lateral vaginal canals are clearly indicated.

The phenominal response of the urogenital-sinus epithelium is characterized by great hyperplasia and by an internal cornification and sloughing of epithelium into the lumen, as well as great extensions in development of the sinus horns which are represented by large solid masses of epithelial cells. A cross-section through the urogenital-sinus region shows the diameter of a cross-section to be nine times that of an untreated female of similar age.

Ser. 115: This female was sacrificed on day 46 after daily treatments with estradiol ointment from day 6 to day 45; it is one of the longer-treated females but is chosen especially for comparison with series 119. These two females, in the same pouch, received identical treatments to day 45; one was preserved to indicate the stage of development 1 day after the last treatment, whereas the second one was permitted to survive without further treatment until day 79.

The condition of female 115 is that of tremendous stimulation of both Wolffian and Müllerian ducts. The normal untreated female of this age shows the Wolffian duct greatly reduced; anteriorly, epoöphoron structures are mostly solid epithelial cords traceable to the rete ovarii. The duct is visible for approximately one-half the distance to the urogenital sinus and is reduced to a solid cord, gradually undergoing atrophy. In contrast, treated female 115 possesses a Wolffian duct which in the anterior region has a well-defined lumen (0.03 mm. diameter); epoöphoron tubules are but little stimulated. Traced caudally, the duct becomes greatly enlarged in size and in the uterine-segment level the Wolffian lumen reaches a diameter of 1.50 mm. and nearer the urogenital-sinus area a diameter of 1.80 mm. Figure 17 (Pl. VI) shows the Wolffian duct imbedded in the walls of the uterus at a level where the diameter of its lumen is 0.2 mm. The duct contains considerable cellular debris, and posteriorly the lumen passes over into the urogenital-sinus region with its tremendous hyperplasia and cornified cell reaction. Thus, not only has estrogenic treatment resulted in the preservation of the Wolffian duct throughout its course, but it has also resulted in causing sufficient growth to produce a lumen of 1.80 mm. in a region in which the duct is totally absent in normal females.

Müllerian ducts in normal females of this age show regional differentiation into an oviduct that is slightly tortuous (lumen 0.03 mm.), a uterine portion somewhat expanded but still lined with a simple epithelium with no evidence of glandular formations (lumen diameter 0.30 mm.), a constricted cervical region (0.07 mm.), and a slightly expanded region for the lateral vaginal canal (0.37 mm), which comes in contact with a solid plug of cells at the urogenital sinus.

The treated female, in contrast, shows tremendous enlargements for the uterine and lateral-vaginal-canal portions but little change in the oviduct portion (aside from more convolutions) or in the constricted cervical canal portion. The uterine portion, despite a growth sufficient to produce a lumen of 2.85 mm. (nine times normal), shows well-developed areas of uterine glands (Pl. VI, Fig. 17); in some areas of the duct, pressures have apparently effectively flattened the epithelium and prevented glandular outgrowths. Lateral vaginal canals are enlarged to a diameter of 2.10 mm. (six times normal), and these open into the greatly hypertrophied urogenital-sinus area. Figure 20 (Pl. VI) shows the transition from the glandular epithelium of the lateral vaginal canal to the hyperplastic urogenital-sinus area, much more apparent at a different level than in this section. Female 119 received identical treatment except that it was not sacrificed until day 79 and hence was without treatment after day 45. Whereas some regression in size of the ducts may have occurred, they are still of essentially the same grade of development as in treated female 115.

In general, such responses as have been described for this youngest and oldest female among the treated group portray the general nature of the modifications produced in young developing females after daily treatments with estrogenic substances during sexual differentiation. Slight individual differences naturally occur, but the notations of lumen diameters in the table roughly express some of these variabilities.

The Wolffian ducts in treated females have, without exception, shown responses not only by their presence at ages when normals have regressed but also by the tremendous growth responses required to produce ducts with lumina up to 2 mm. in diameter in sectioned materials. Frequently a duct on one side may be much greater than the opposite, but no selective laterality in response has been noted. Considerable differences exist also in the amount of cellular debris present in the lumen. Stimulation of the epoöphoron region has not been conspicuous, but decided stimulation has been evident in posterior mesonephric tubules (paraoöphoron) in females at ages when normally these would be reduced to solid degenerate remnants. In older females, in particular, the caudal end of the duct is frequently seen to be thrown into pronounced folds, exhibiting considerable grades of metaplasia, as if linear growth tendencies have been unable to express themselves.

Müllerian ducts in estrogen-treated females have responded invariably with pronounced growth; this has involved precocious regional differentiation, precocious glandular developments, and great increase in size. Inherent regional tendencies express themselves as they would at later ages in untreated normal animals, since, if treatments were initiated prior to the Müllerian duct contact with the urogenital sinus, the simple tube still responds with a distinct oviducal and uterine portion. The two youngest females listed in Table 2 did not show distinctly a lateral vaginal canal region, but by the 22d day (ser. 100) this was well indicated, and the constricted cervix portion connected the uterus and lateral vaginal canal.

Casual study of ovaries has failed to reveal outstanding modifications, but normal ovarian development and hormonal responses of this organ are being investigated by Mr. Charles Morgan.

For the purpose of contrasting differential responses in the two sexes to treatments with estrogenic substances, it is desired to call attention to three pairs of embryos representing a male and a female, in the same pouch, that had received identical treatments.

Male 49A and female 49B were treated on days 5-16 and sacrificed on day 17. It can be seen from Table 2 that Wolffian ducts in the female were approximately four times as large as those in the male and that female Müllerian ducts were approximately double those in the male. Male 55A and female 55B which were treated from day 5 to day 22 and sacrificed on day 23 show that the Wolffian ducts were actually somewhat smaller in the male than in the female, but the Müllerian ducts in the female were again much more responsive than those in the male. The pair, male 109 and female 108, treated from day 6 to day 29 and sacrificed on day 30, reveal Wolffian ducts in the female approximately fifteen times the diameter of those in the male and Müllerian ducts approximately thirteen times greater in luminal diameter in the female. This selective tendency of both sets of ducts in females to respond more vigorously than in males in the same pouch and receiving identical treatments is further emphasized by a glance at the measurements recorded in the table for the group as a whole.

From such comparisons it becomes evident that the homologous ducts respond differently in the two sexes to the same treatment. The genetic constitution appears to establish a foundation for differential reactivity to the same chemical treatment, even though treatments are given continuously from a period prior to definite embryological formation of the ducts.

In résumé, estrogenic treatments of developing opossums have induced growth in external genitalia of both sexes, but the phallus differs qualitatively from that produced by androgens. Wolffian ducts have been stimulated in both sexes, but more intensely in females; they have been preserved from disappearance and increased greatly in size. Müllerian ducts have been stimulated in both sexes, moderately in males, tremendously in females; in males ostium and oviduct are usually not preserved, and the tube is blind at both ends, while in females precocious regional differentiation, general growth, and glandular differentiation have been evident. The prostate gland in males is suppressed or, if already formed, is converted into an atypical hyperplastic mass. The urogenital sinus is very sensitive to estrogenic effects and undergoes tremendous hyperplasia and metaplasia. Gonads are not markedly modified.

VI. ADMINISTRATION OF GONADOTROPIC SUBSTANCES

The gonad-stimulating principle of pregnant mare serum (equine gonadotropin) has been administered to both male and female opossums at different periods in development. Provided the gonads would respond by precocious or accelerated sex-hormones secretion, the results on the various end-organs should be most instructive in comparison with responses of the same organs to the chemical hormones that have been applied from without. Furthermore, since the gonads of many species are remarkably stimulated to excess hormone secretion, especially after the attainment of a definite stage in maturity, it was anticipated that perhaps some suggestion might follow with regard to the age at which gonads first begin secreting their respective hormones, or at least when they were definitely capable of doing so. If this should prove to be the case, we would be provided with a means by which to determine the onset of secretion under normal conditions.

This phase of the study must be regarded as the most incomplete, since the number of developing young available for this purpose was small. Table 3 gives a list of 9 individuals which received treatment, recording age, type of hormone, and duration of the injection period. Gonadogen and gonadin are each the concentrated gonadotropic principle from pregnancy serum. Daily injections of 1 rat unit of gonadogen into rats for a 17-day period, terminating with a body weight of 60 gm., revealed a thirty-times increase in fresh weight of seminal vesicles over normal controls; similar daily injections of 5 rat units of gonadin (different unitage) produced an increase of twenty to twenty-five times in seminal-vesicle weight. In 30-day female rats similar dosages of each preparation produced an increase in the fresh weight of the uterus approximately ten times that of the control female. The body weight of treated opossums was in the range of 3–4 gm. at the end of treatment for younger stages and of 130 gm. for the 100-day specimens: excessive amounts of these hormones were thus given younger opossums.

A. *Males.*—No effects from the treatment were perceptible in the 17-day and 21-day males. Careful examination of testes, Wolffian ducts, Müllerian ducts, prostate gland,

and urogenital sinus, in comparison with similar aged normal males, reveals no detectable modification. In the 30-day male (ser. 112) prostate-gland and Cowper's-gland differentiation on a subjective basis appeared to be slightly stimulated; actual microscopical measurements, however, failed to reveal any very evident increases in total cross-section of the urogenital sinus and prostate outgrowths, although the length attained by prostate cords appears to be slightly greater than in a similar-aged normal control. Müllerian ducts or Wolffian ducts were not perceptibly modified. If this is really a case of response, as it appears to be, the stimulation is but very slight. The 100-day male (ser. 84) reveals definite, though slight, acceleration in prostate differentiation. The central ends of prostate cords, at their attachment to the urogenital-sinus epithelium, show more definite luminal formation than in the control. Differentiation, however, has not approached the stage of puberal development. The weight of one testicle of this treated male, in comparison with an untreated littermate, was approximately 60 per cent heavier, but there is no apparent histological difference between the two organs.

TABLE 3

GONADOTROPIC TREATMENT OF OPOSSUM POUCH YOUNG

Ser	Age at Autopsy (Days)	Daily Treatment
	Males	
99A	17	10–16 gonadogen, 1 rat unit
102A	21	10–20 gonadogen, 1 rat unit
112	30	15–29 gonadin, 5 rat units
84	100	85–99 gonadogen, 5 rat units
	Females	
99B	17	10–16 gonadogen, 1 rat unit
102B	21	10–20 gonadogen, 1 rat units
113	30	15–29 gonadin, 5 rat units
116	38	15–37 gonadin, 5 rat units
90	100	85–99 gonadogen, 5 rat units

Gonadotropic treatments have given but very slight evidences of effectiveness in stimulating the testicle of developing male opossums to secrete hormones; and until other material is available for study, the limitations of effectiveness will have to remain uncertain.

B. *Females.*—A study of the 5 treated females listed in Table 3 is summarized by the statement that no effects have been observed from the treatments given. Younger specimens carefully examined for Müllerian and Wolffian duct and urogenital-sinus modification have revealed no responses. Even in the 100-day treated specimen a study of the ovary fails to elicit any detectable response; no suggestion of antrum formation in follicles is evident.

VII. DISCUSSION

The study which has been conducted on the development of the reproductive system of the opossum, and the unusual aspects of it that follow treatment with sex hormones, has involved several somewhat special questions, some of which may be formulated as follows: (1) Does the differentiating reproductive system respond to treatments with androgenic, estrogenic, or gonadotropic substances, and, if so, how early do such effects become apparent? (2) Can artificially introduced chemical hormones effectively override zygotic sex-differentiating factors? (3) Is there specificity in response of developing structures to these chemical substances—male structures being stimulated by androgens and female structures by estrogens? (4) What bearing do observations have on the question of hormone antagonism? (5) How do effects from such treatments with chemical hormones compare with results noted in freemartins? (6) From the nature of responses, if any, what suggestions appear relative to the possible role of such substances in normal sex differentiation, or the possible part played by hormones secreted by developing gonads? Additional supplementary questions have presented themselves, but the essence of the inquiry is perhaps sufficiently laid before us for consideration.

1. Attention to the preceding pages can leave no doubt that both the developing male and female reproductive systems are subject to modifications by application of pure chemical androgens or estrogens. In point of time, responses can occur early, since at the 15th or 16th day in the pouch, after 11 days of treatment, some modifications have become evident. At later periods in development and with fewer days of treatment, much more marked modifications are apparent, which suggests that a certain degree of differentiation is required before responses occur; to determine at just what point, in terms of maturity of the developing system, the ducts become sensitive requires additional experimentation.

In point of character, the responses have all been of the nature of stimulations rather than inhibitions, and no clear instance of inhibitory phenomena has been apparent. One could perhaps speak of prostate-gland inhibition in males by estrogens, since under such treatments the prostatic outgrowths from the urogenital-sinus epithelium fail to become evident. However, the actual response of this region is characterized by great stimulation of cell division, and hyperplasia, in the urogenital-sinus epithelium; and it appears more accurate to regard the end-result as an example of competition between two different types of stimulated cell response—peripheral cellular growth for prostatic cord formation, and proliferation centrally, giving the high degree of hyperplasia and accompanying metaplasia; certainly, the total amount of epithelium is much increased over normal when estrogens are applied, hence inhibition could only be held for the *type of differentiation*. The region, and actual source of prostate cells, has been greatly stimulated and not inhibited, even though the course of development has been turned in a different direction.

A second modification capable of being interpreted as inhibition is that of the phallus after estrogenic treatment. But here again, as with the prostate, there has been real stimulation of this structure and not an inhibition; the total mass has been markedly increased, but its development has followed a different course from that dictated by its genetic constitution.

Since these two mentioned conditions are thus interpreted as stimulations rather than as inhibitions, the entire results appear to be devoid of any apparent inhibitory action.

One somewhat unexpected result has been the differential sex response. Female tissues are more responsive to either chemical androgens or estrogens than are those of the male; hence one must admit at the outset that zygotic sex-differentiating factors have laid the basis for differential responses by homologous structures. Thus, Müllerian ducts in the two sexes have different fundamental capacities to respond to treatment. It is to be remembered that both androgens and estrogens have been applied continuously (daily) since a stage in development when the duct was in progress of formation and continuing its growth toward the urogenital-sinus connections. At all ages of sacrifice the Müllerian ducts in the male failed to be maintained at either the ostial or the urogenital-sinus end, and the intermediate portions that remained responded weakly to such treatments as those which induce enormous responses of the same structure in the female. Since pairs of embryos in the same pouch receive identical treatment, sex is the only differential; hence, it is readily apparent that the growth and development of the Müllerian duct is by no means entirely controlled by the chemical androgens applied.

Essentially similar conditions hold for Wolffian ducts, since these structures in the female are somewhat more responsive than those of the male; in fact, Wolffian-duct response in males to androgens is remarkably unimpressive, and in the final state, though admittedly somewhat atypical in growth, Wolffian ducts in females usually exhibit much greater response to androgenic treatments than do those in males. The same is even more apparent when estradiol is administered; much larger Wolffian duct remnants are found in females than in males.

2. The question whether introduced chemical hormones override zygotic sex-differentiating tendencies may be answered by stating that, since inhibitory tendencies are not apparent, the changes revealed are those of addition of structures to those usually present. Thus, androgens applied to males cause little, if any, modification, except the intensification of development in the prostate gland and external genitalia; in females these substances induce prostate development, increase the size, and change the form of the phallus and favor retention of Wolffian ducts (normally degenerate). With regard to estrogens, the zygotic differentiating tendencies in females are tremendously supplemented, and Wolffian ducts are added in whole or in part. In males there is also a tendency to intensify development of the ducts by estrogenic treatment, since in general the Wolffian ducts exhibit a greater luminal diameter than in a similar-aged normal male; derivatives of the Müllerian duct (usually degenerate) have been maintained and may exhibit considerable response; this response is decidedly less marked in males than in females. It is clear that, in the main, zygotic sex-differentiating factors remain in ascendancy; but some redirection, or intensification, of differentiation may appear. Inhibition of heterologous characters by chemical hormones is not apparent.

3. The question of specificity of response to homologous hormones brings forth rather unusual comments, since in the majority of reports in the literature, aside perhaps from some cases in mammals, homologous responses are emphasized.

It has been repeatedly pointed out above that both sets of embryonic sex ducts show responses to treatments with either androgens or estrogens. In females an outstanding result has been the responses of Müllerian ducts to androgens (see, e.g., Pl. IV, Fig. 6, and Pl. V, Fig. 8). Whereas this female duct in males shows some responses to androgens by its persistence over a considerable proportion of its natural course, we see that in pairs of male-female such as 101A and 101B, which have received treatments continuously from a period in which formation is just under way, the Müllerian duct in males shows a maximal luminal diameter of 0.01 mm., whereas in the corresponding female this may be more than one hundred times as great. Furthermore, Wolffian ducts, which we might anticipate would respond, do so only slightly in males and with somewhat greater intensity in females. Similarly, with regard to specificity, estrogenic treatments of females are frequently followed by Wolffian ducts as large as or larger than Müllerian ducts. Again, response of Wolffian ducts (unexpected) is greater in females than in males. It is evident, therefore, that specificity in response to chemical hormones does not characterize the developing embryonic sex ducts in the opossum.

4. The question of hormone antagonism in development has been covered in part already. It may be restated that chemical androgens do not suppress or inhibit differentiation of the female reproductive system but rather speed up and intensify development and exhibit precocious differentiation in the Müllerian system. In a similar manner estrogens have not shown antagonistic influences in developing males, with the possible exception of the prostate and phallus regions, where the type of differentiation is changed—they serve merely to add certain female rudiments that are not normally retained. Rather than inhibiting heterologous characters, these chemical hormones proved to exert a stimulating influence on opposite sex characters.

5. Before making direct comparison between the results of chemical-hormone treatment of opossums and conditions in freemartins, it is perhaps justified to review some points in the original interpretations of the freemartin condition in the light of twenty-three years' additional experience. The facts portrayed by Lillie have never been questioned, and from 1917 to the present his suggestive interpretations have withstood the rigors of numerous experimentations conducted over the world. It is to be recalled that several necessary postulates had to be proposed before the theory of hormone actions could be logically developed. At least a few of these may be mentioned.

a) The hormone theory of embryonic duct differentiation presumes that the differentiating testicle or ovary secretes a hormone that guides differentiation of the corresponding reproductive system, although Lillie mentions the possibility that the circulating substances from the male—effectively inducing modifications in the female—might have a different origin than the testis. It appears that substantial evidence is still lacking that a gonad secretes effective morphogenetic substances, or sex hormones, during the course of its early differentiation, when these would have to act to guide development of the duct system. No one has yet obtained an embryonic hormone. And, although Womack and Koch (1930) obtained comb-growth-stimulating substances from extracts of testes from calves in late fetal stages, such active material was not obtained from extracts of urine from postnatal calves. It is true that for amphibia it is generally accepted that an inductive substance (medullarin or cortexin, of Witschi) does issue from the developing gonad, but this is believed to be not a circulating hormone but something different from hormones secreted by adults. Thus, in comparing effects in females treated with chemical hormones with effects in freemartins, one must keep in mind the possibility that these effects are produced by entirely different active substances. Despite the inclinations of Burns (1938*b*) to consider it possible that the same substance is active throughout an entire ontogeny, the evidence from these investigations on opossums gives indications of a different nature.

b) Steinach, prior to 1917, had claimed a mutual antagonism between sex hormones of the nature that the presence of one hormone inhibited development of the opposite sex characters, and Lillie's demonstration of an inhibition of the ovarian cortex and Müllerian ducts in freemartins by substances transferred in the blood of the male would appear to lend credence both to the antagonism hypothesis advanced by Steinach and to the idea that the freemartin effects were due to a sex-hormone action. More recent studies have failed to substantiate Steinach's conceptions of hormone actions, however, and injections of chemical hormones have shown but slight inhibiting effects on sexual differentiation in the heterologous sex.

c) The explanation of the freemartin condition on the basis of the hormone theory must presume a sex specificity in response of homologous tissues to the hormone involved. The results noted on the opossum and on embryos of other species have failed to indicate such specificity of response to pure chemical hormones.

d) Modifications in the freemartin failed to involve the external genitalia, since these remained entirely female even in those specimens showing the highest grades of internal modification; hence, phallic development appeared to be the least liable to modification. Chemical-hormone application, however, has revealed the developing external genitalia to be very sensitive to modifying influences—a point stressed also in the work of Burns (1939*d*, 1939*e*).

e) Lillie recognized the problem of accounting for the protection of a developing male embryo from modifying influences of maternal hormones and suggested placental impermeability to maternal hormones as a possible protective mechanism, although other possibilities were noted. It has been shown by later observations that amniotic fluid in some species is a rich source of estrogenic substances, and normal male development is not influenced by their presence.

With these points in mind one may suggest rather directly the observations on agreement and disagreement between conditions resulting from chemical-hormone treatment of the opossum and the freemartin conditions in which, theoretically, a male hormone secreted by the embryonic testis was supplied to the female. The comparison involves only chemical androgens in females, since theoretically no estrogenic substance was present in the freemartin, owing to early suppression of ovarian differentiation; hence, the male showed no effects from ovarian secretions.

Organ	Freemartin	Female Opossum
Ovary	Cortical inhibition	No apparent effect
Müllerian duct	Inhibition	Stimulation
Wolffian duct	Retention and stimulation	Incomplete retention, mild stimulation
Prostate	Stimulation	Induction and stimulation
External genitalia	No effect	Marked stimulation in male direction

A consideration of such a diverse set of responses in the cases of these 2 females of different species to male hormones suggests such interpretations as the following: (1) species reactions are markedly different in opossum and cattle sex differentiation, (2) supply of effective substance to the modifiable system is of distinctly different order, or (3) entirely different substances are concerned in producing the observed effects. It will appear evident, with the above points in mind, that freemartin effects have not been produced in the opossum by treatments of developing young with chemical sex hormones.

6. A consideration of the influences of chemical hormones on sexual differentiation in

the young of opossums provides little or no suggestion that such substances play any role in normal sex differentiation. Prevailing ideas relative to hormonal control of sexual differentiation imply, in general, a high degree of similarity in capacity for homologous structures in the two sexes to respond with homologous stimulation and heterologous inhibition.

Experiments reported here have amply demonstrated a decided difference in the capacity of homologous ducts to respond to a given treatment, especially in the case of Müllerian ducts. Likewise, the lack of specificity in response on the part of the sex ducts during developmental stages would seem to preclude the effective operation of these hormones or other estrogenic or androgenic substances having a similar effect. Thus, amounts of androgens in males that stimulate Wolffian ducts cause retention and stimulation of residual portions of Müllerian ducts in whole or part. Whereas it is probably true that a greater dosage has been experimentally administered than would be present under normal conditions, one may presume that the suggested heterologous inhibition would be even greater than in normal development. The reverse, however, is true.

The inability to see suggestions from these experiments that would justify the idea of a hormonal control on duct differentiation, or to see that the results lend credence to the freemartin interpretations involving gonadal secreted hormones, have led to an attempt to suggest an interpretation that might plausibly cover both phases of development. Prior to making the suggestions, it appears to be advisable to review very generally the effects that have been noted from treatments of other species during developmental stages with such chemical hormones as have been employed.

Among amphibia (Burns, 1938, 1939; Ackart and Leavy, 1939; Foote and Witschi, 1939; Foote, 1940; Puckett, 1940) specificity of response has largely followed when chemical-hormone treatment has been carried out during early developmental stages, both as regards ducts and, to some extent, gonads. Where mention is made of effects on duct development, Wolffian ducts but not Müllerian have responded to androgens; and Müllerian, but not Wolffian, ducts have responded to estrogens. Burns (1939*a*) has mentioned inhibitory effects on Müllerian ducts by testosterone. Gonadal development has usually been modified in a specific sense in that androgens have favored testicular development in genetic ovaries but have been largely without effect on genetic testes, and estrogens have led to the development of genetic testes into ovaries but have shown no appreciable effects on genetic ovaries. In slowly metamorphosing forms (*Rana catesbiana*) Puckett (1940) found gonadotropic agents a necessary aid to the reactions before the sex hormones could exert their effects, since androgens or estrogens alone failed to effect gonadal modifications.

Among reptiles Risley (1937) found evidences in turtles of androgenic stimulation of ovarian medulla without inhibition of cortex, but duct responses were not emphasized. Sexually differentiated, but immature, alligators did not react so specifically to sex hormones. Androgens had no effect on Wolffian ducts of either sex, but Müllerian ducts in females were markedly stimulated. Estrogens stimulated markedly the Müllerian ducts of females and, to a lesser extent, residual portions of this structure in males but exerted no effect on Wolffian ducts. Gonads exhibited responses to estrogens by hypertrophy of ovarian cortex and any residual cortex on testes but was without effect on ovarian medulla or upon the testis proper (Forbes).

The bird exhibits a variety of responses when treated with androgens and estrogens during sexual differentiation, on account of, first, an inherent asymmetry and, second, differential responses to androgens of slightly different chemical constitution (Dantchakoff; Kozelka and Gallagher; Wolfe and Ginglinger; Willier, Gallagher, Rawles, and Koch; Willier). In most cases a high grade of specificity was exhibited in duct responses —Wolffian ducts responding to androgens and Müllerian ducts to estrogens; but Müllerian ducts of females are not inhibited by testosterone—although they do show such a response to androsterone—and Wolffian ducts are not inhibited in males by estrogens. Exceptions to this general statement are registered by Willier, Rawles, and Koch (1938), who found that Müllerian ducts in males show persistence and stimulation after treatments with androsterone and dehydroandrosterone, whereas in females they show no response.

Gonadal responses in the bird to sex-hormone application have been decidedly variable, and for detailed consideration the reader is referred to the account of Willier (1939). In brief, testosterone exerts no appreciable effects on testes or ovary, but androsterone stimulates incipient cortex on the left testis and may change the internal portion to a typical ovarian medulla (ovotestis); the right testis may likewise be modified by a large dosage of androsterone into an ovarian medulla (cortex absent). Neither of these two chemical androgens appreciably affects the developing genetic ovary. Estrogens, while showing practically no modifying effect on genetic ovaries, exert an effect on testes similar to androsterone (left, ovotestis; right, ovarian medulla).

Among mammals, hormonal treatment during sexual differentiation has been employed on rodents (*guinea pig:* Dantchakoff; *rat:* Greene, Burrill, and Ivy; Hamilton and Gardner; Hamilton and Wolfe; Moore; *mouse:* Raynaud; Turner) and on the opossum (Burns; Moore). Since no pronounced qualitative modifications of gonads have followed either treatments with androgens or estrogens in any of these species, our attention may be limited to the question of specificity of reaction on the part of sex ducts. Generally speaking, androgenic treatment has revealed strikingly little effect on Wolffian-duct differentiation in males, but in females the duct has shown a very consistent persistence; and such outgrowths as seminal vesicles have been found in female mice and rats. Expected inhibitory effects of androgens on Müllerian ducts have not been apparent, but instead these ducts have been retained in all treated female rodents and in the male opossum; and they have been tremendously stimulated in developing female opossums. In rats and mice the distal vagina has been consistently absent after androgenic treatments, suggesting a specific inhibitory effect, but this urogenital-sinus derivative is inhibited to a similar degree occasionally in untreated young (Plagge and Lamar, 1939) and also by estrogenic treatments (Greene, Burrill, and Ivy, 1939*b*). Because of these facts it would appear justified to consider the absence of terminal vagina as a nonspecific retardation of normal developmental processes that could conceivably be brought on by many different agents rather than by a specific hormonal inhibition of heterologous characters.

Estrogenic treatment during sexual differentiation has been carried out on the rat by Greene, Burrill, and Ivy (1939*b*) and on the opossum by Burns (1939*c*, 1939*e*) and Moore (1939). Such treatments of females would be expected, on the basis of the theory of hormonal differentiation of sex ducts, to show only possible stimulation of the Müllerian system; but in the rat it was noted by Greene and associates that complete retention of Wolffian ducts provided as complete a male system of excurrent passages as were present in normal males; seminal vesicles were not noted. In the opossum, likewise, the Wolffian ducts of females were stimulated by estrogens rather than inhibited.

When one thus contemplates such an array of results as have emerged from treatments of animals undergoing sexual differentiation with androgens and estrogens, it becomes a real difficulty to accept in principle the contention that specific hormonal substances issuing from developing gonads are the controlling agents in differentiation of the reproductive system. The postulated stimulation of homologous characters and inhibition of heterologous characters does not hold.

In an attempt at a reorientation to this complex problem, four important aspects have been taken into consideration.

1. One of the more impressive aspects of this study has been the different capacity of the same embryonic structure in the two sexes to respond to the same treatment. It is to be remembered that treatments were initiated during the development of these structures and not subsequent to their differentiation. It is apparent that a Müllerian duct in a female and one in a male have strikingly different capacities to react to the same treatment. When estrogens are applied alike to females and males in the same pouch, female Müllerian ducts respond with a tremendous development in comparison with those in males. Similarly, the application of androgens to both sexes emphasizes the capacity of female Müllerian ducts for a greater response than is evidenced in male Müllerian ducts. The Wolffian duct, though showing less spectacular responses especially to androgens, proves to have different capacities to respond to those hormones despite the fact that these ducts have functioned equally in both sexes as a urinary outlet from the mesonephros during a considerable portion of the treatment. There is thus to be emphasized a zygotic differential in capacity of basic embryonic tissue for response. Burns (1939*d*, 1939*e*) has likewise stressed different capacities of the same structure in the two sexes to respond to a given treatment.

2. Our current viewpoint permits the assumption that primary sex-differentiating factors, the zygotic sex differentials, are capable of converting the undifferentiated gonad into an ovary or testis, as is proper on account of the original determination. There does not appear to be any known fact against the conception that duct differentiation or other structures (prostate gland, Cowper's gland, phallus, etc.) may likewise depend upon the same forces that molded the proper gonad development, and that duct development is parallel to gonad development, perhaps dependent upon the same differentiating factors rather than being conditioned to develop by hormones produced by these developing gonads.

3. The fact is well established that the sex ducts are capable of showing response to chemical hormones even during the course of their development. However, this need not necessarily be interpreted as evidence that their normal development depends upon such hormones. There is no longer doubt that their fundamental functional differentiation approaching puberty, and in subsequent periods, depends upon gonad-hormone secretion; but the point at issue is the period in ontogeny at which this gonadal control takes hold. Since residual tissues respond to hormones, and in these experiments give no evidence of heterologous inhibition, it is difficult to conceive how one set of ducts could survive and the other disappear if their development depended upon a sex hormone of the nature employed in these experiments; each set responds to either androgen or estrogen administration.

4. It is yet unknown at what period in gonad development hormones are first secreted; our methods are scarcely adequate to make this determination. One fact may be suggestive: gonads that are actively secreting hormones, and even prior to this demonstrable

activity, have shown, in practically all instances, a response to gonadotropic substances. If the assumption is valid that gonads capable of secreting hormones will respond to gonadotropic substances—and all previous experiments indicate that equine gonadotropin is highly similar to pituitary materials in its action—it should follow that, since gonadal response in opossums has been negative in early stages and very weak or absent even up to the 100-day period, the gonads during the stage of duct development are not secreting hormones.

With such considerations in mind, the following relatively simple working hypothesis of sexual differentiation in the opossum may be formulated.

Sexual differentiation involving gonads, as well as ducts, phallus, etc., are under the control of genetic sex-determining factors and not controlled by gonad-secreted hormones. It is possible that, in addition to the genetic factors in the localized anlage of reproductive organs, certain stimulating humoral substances issue from the entire somatic complex of the organism; and that these, rather than specific secretions elaborated by the developing gonad, favor homologous duct development. It may follow from this—and in keeping with the freemartin it would be expected - that the genic constitution of the tissue or organ in one sex could conceivably be overridden by such somatic humoral substances from the opposite sex and differentiation be redirected. Thus, on this basis, the undoubted humoral transmission of substances from the male twin to the freemartin would merely suggest that the unknown effective substances issue from the entire somatic complex of the male rather than from its developing testes. Therefore, there is postulated not only an effective substance different from sex hormones of later life but a substance that is produced in the organism as a whole rather than in the gonads. This does not deny an activity to gonad-secreted hormones on sexual equipment after its differentiation, but it does suggest that such hormones, later certainly effective, probably have no place in controlling early differentiation of the essential equipment of the system.

Experiments designed to test the necessary corollaries of such a conception are under way but are thus far too incomplete to warrant discussion. A tendency to conceive of a mechanism involved in sexual differentiation different from that generally current, sometimes designated as the "dihormonic theory," is already evident in Weisner's (1935) development of a "monhormonic" theory. In contrast, the suggestions here offered might be labeled "anhormonic"; but it is considered premature to make a commitment with regard to hormonal substances. It appears probable that humoral substances do play a part in giving expression to inherent potencies carried by the genetic constitution of the tissues; and these substances, if real, may ultimately be considered to be hormones. But the evidence suggests that, whereas in the later physiology of the reproductive system gonad-secreted hormones probably play the leading conditioning role, one should not deny the possible operation of a different humoral mechanism, provided, perhaps by substances produced by the whole embryo instead of by the developing gonads.

In point of time, the limits of operation of the nongonadal and gonadal humoral substances are ill defined and must await a determination of the time of appearance of effective gonadal secretions. That the nongonadal humoral factors may operate well after birth and may be decidedly overlapped by the gonadal secreted substances appears to be indicated by the work of Price (1936, 1939, 1941) on the prostate gland of the rat. Since remarkable species differences exist and since any biological reaction is dependent upon the capabilities of tissue responses as well as upon the inciting agent, one must expect differences in time, degree, and capacity of response. Relative to the basic freemartin responses, Lillie's portrayal of which has provided the stimulus for so much commendable effort in this field in the last 23 years, it should be pointed out that Wislocki (1939) has revealed a condition in marmoset twinning that should provide a duplication of the condition occurring in cattle without the appearance of any suggestion of similar responses.

VIII. SUMMARY AND CONCLUSIONS

A. 1. The newborn opossum is sexually undifferentiated and possesses a functional mesonephros for approximately 2 weeks.

2. Pouch young become fixed on the nipples and retain an uninterrupted attachment for 60–70 days.
3. Forty stages of normal reproductive-system development, involving both sexes, have been studied in serial section to include the period between days 1 and 100.
4. Gonad differentiation suggests ovaries or testes by the third day.
5. Müllerian ducts appear by day 3 and reach the urogenital sinus at approximately day 10. In females further development produces by day 30 a definite regional differentiation into oviducts, uterus, cervix, and lateral vaginal canals. In males these ducts atrophy progressively in a posteroanterior direction subsequent to day 10. Small anterior rudiments remain along the epididymis and exhibit increased growth from about day 80; by day 100 they are short, luminate tubes.
6. Wolffian ducts exhibit retrogression on termination of urinary function in both sexes. In females the ducts atrophy progressively in a posteroanterior direction; epoöphoron tubules are conspicuous.
7. The prostate gland arises in the male as epithelial buds from the urogenital-sinus epithelium along its course on day 16; in females, the prostate anlage does not appear at any stage of development. Prostate buds appear in males by day 15; branchings of original outgrowths occur about day 40; by day 100 the branching cords permeate the mesenchyme of the urogenital-sinus wall and constitute the foundations for future glands; by the 8th month central ends of the glands are canalized; and the adult type of inclosed prostate is present at 1 year of age.
8. Testes show little spermatogenetic activity up to the 100th day; by 1 year spermatozoa are being produced. Ovaries at the 100th day contain numerous follicles with large ovocytes, but no antra.

B. Applications of androgenic substances were made by ointment, or by injection, in both sexes, covering periods from day 3 to day 100 and in a few older individuals. Serial sections of the reproductive system were prepared of 18 males and 22 females treated with androgens.

1. Androgens exert only mild stimulation on Wolffian ducts in males and a somewhat greater effect on female Wolffian ducts; these latter are preserved in females in an atypical manner.
2. On Müllerian ducts mild effects are visible in males, extreme stimulation occurring in females. Precocious development of the female tract involves growth, regional differentiation, and glandular developments. Cessation of treatment does not readily involve regression.
3. Prostatic development in males is markedly hastened. In females, induction of prostate formation occurs, as well as precocious stimulation of growth and differentiation.

  4. Testes and ovaries are apparently little affected by androgenic treatment.
  5. Phallic stimulation is pronounced in both sexes; precocious differentiation is readily induced. The female phallus is converted into a typical penis with bifurcate glans.

C. Application of estrogenic substances to both sexes involved skin ointments and subcutaneous injections. Estrogenic substances are decidedly toxic and caused the death of many treated young. Serial sections of the reproductive system were prepared of 8 treated males and 11 estrogen-treated females.
  1. Wolffian ducts in both sexes have been stimulated, but more appreciably in females than in males.
  2. Müllerian ducts in males are not maintained in normal relations, but localized remnants become large luminate segments. In females the ducts are precociously stimulated, and glandular differentiation greatly hastened.
  3. The urogenital sinus is especially sensitive to estrogenic substances; the epithelial lining, in both sexes, exhibits tremendous hyperplasia and metaplasia, and cornified cells are sloughed off into the lumen.
  4. Prostate development in males does not occur, for the resulting cell migration is directed centrally rather than peripherally; previously formed prostate outgrowths are converted into atypical hyperplastic and metaplastic growths of enormous proportions.
  5. Ovaries or testes are not appreciably modified in their development.
  6. External genitalia are increased in size by estrogenic treatments, but the type of growth is decidedly different from that following androgenic substances; it is an increase in all directions, resulting in a rounded projecting prominence rather than the typical elongate penis type.

D. Zygotically different responses have been noted from the fact that—
  1. Wolffian-duct stimulation by androgens is relatively greater in females than in males;
  2. Müllerian ducts in males show comparatively little response to androgens, whereas in females tremendous precocious stimulation results from treatment;
  3. Wolffian ducts in males respond less markedly to oestrogenic substance than do those in females;
  4. Müllerian ducts are not retained in their normal relations in males, whereas in females precocious differentiation occurs.

E. Quite similar responses follow in each sex in (*a*) androgenic effects on the prostate, though induction occurs in females and (*b*) on external genitalia; and in (*c*) estrogenic effects on the urogenital sinus and on external genitalia.

F. Gonadotropic agents evoke little or no response at early ages, and further work is required to demonstrate the capacity of gonads to secrete hormone in the early stages of their development.

G. Reasons are given for suggesting that early duct differentiation is not dependent on gonad-secreted hormones, and a tentative working hypothesis is suggested.

LITERATURE CITED

Ackart, R. J., and Leavy, S. 1939. Experimental reversal of sex in salamanders by the injection of estrone. Proc. Soc. Exper. Biol. and Med., 42:720–24.

Baxter, James S. 1935. Development of the female genital tract in the American opossum. Carnegie Inst. Publ. No. 459, Contr. to Embryology, 25, No. 145, 17–38.

BISSONNETTE, T. H. 1924. The development of the reproductive ducts and canals in the free-martin with comparison of the normal. Amer. Jour. Anat., **33**:267–345.

BURNS, R. K., JR. 1930. The process of sex transformation in parabiotic *Amblystoma*. I. Transformation from female to male. Jour. Exper. Zoöl., **55**:123–69.

———. 1931. II. Transformation from male to female. *Ibid.*, **60**:339–87.

———. 1938*a*. The effects of crystalline sex hormones on sex differentiation in *Amblystoma*. I. Estrone. Anat. Rec., **71**:447–67.

———. 1938*b*. Hormonal control of sex differentiation. Amer. Nat., **72**:207–27.

———. 1939*a*. The effects of crystalline sex hormones on sex differentiation in *Amblystoma* II. Testosterone propionate. Anat. Rec., **73**:73–93.

———. 1939*b*. Effect of testosterone propionate on sex differentiation in pouch young of opossum. Proc. Soc. Exper. Biol. and Med., **41**:60–62.

———. 1939*c*. Effects of female sex hormones in young opossums. *Ibid*, pp. 270–72.

———. 1939*d*. The differentiation of sex in the opossum (*Didelphys virginiana*) and its modification by the male hormone testosterone propionate. Jour. Morph., **65**:79–119.

———. 1939*e*. Sex differentiation during the early pouch stages of the opossum (*Didelphys virginiana*) and a comparison of the anatomical changes induced by male and female sex hormones. *Ibid.*, pp. 497–547.

CHASE, ELIZABETH BROWN. 1939. The reproductive system of the male opossum, *Didelphys virginiana* Kerr, and its experimental modification. Jour. Morph., **65**:215–39.

DANTCHAKOFF, VERA. 1936. Réalisation du sexe à volonté par inductions hormonales. I. Inversion du sexe dans un embryon génétique mâle. Bull. biol., **70**:241–307.

———. 1937. Sur l'obtention expérimentale des free-martins chez le cobaye et sur la nature du facteur conditionnant leur histogenèse sexuelle. Compt. rend. séanc. Acad. sci., **204**:195.

FOOTE, C. L. 1940. Response of gonads and gonaducts of amblystoma larvae to treatment with sex hormones. Proc. Soc. Exper. Biol. and Med., **43**:519–23.

FOOTE, C. L., and WITSCHI, E. 1939. Effect of sex hormones on the gonads of frog larvae (*Rana clamitans*). Sex inversion in females; stability in males. Anat Rec., **75**:75–83.

FORBES, T. R. 1938*a*. Studies on the reproductive system of the alligator. II. The effect of prolonged injections of oestrone in the immature alligator. Jour. Exper. Zoöl., **78**:335–67.

———. 1938*b*. III. The action of testosterone on the accessory sex structures of recently hatched female alligators. Anat. Rec., **72**:87–95.

———. 1939. V. The effect of injections of testosterone propionate in immature alligator. *Ibid.*, **75**:51–57.

GREENE, R. R.; BURRILL, M. W.; and IVY, A. C 1939*a*. Experimental intersexuality: the effect of antenatal androgens on sexual development of female rats. Amer. Jour. Anat., **65**:415–69.

———. 1939*b*. Experimental intersexuality. The paradoxical effects of estrogens on sexual development of the female rat. Anat. Rec., **74**:429–38.

HAMILTON, J. B., and GARDNER, W. U. 1937. Effects in female young born of pregnant rats injected with androgens. Proc. Soc. Exper. Biol. and Med., **37**:570–72.

HAMILTON, J. B., and WOLFE, J. M. 1938. The effect of male hormone substances upon birth and prenatal development in the rat. Anat. Rec., **70**:433–40.

HARTMAN, CARL. 1923. The oestrous cycle in the opossum. Amer. Jour. Anat., **32**:353–421.

HILL, J. P. 1900. On the foetal membranes, placentation and parturition of the native cat (*Dasyurus viverrinus*). Anat. Anz., **18**:364–73.

HUMPHREY, R. R. 1929. Studies on sex reversal in *Amblystoma*. II. Sex differentiation and modification following orthotopic implantation of a gonad preprimordium. Jour. Exper. Zoöl., **53**:171–219.

———. 1935. VII. Reversal of sex type in gonadic preprimordia of *A. punctatum* males implanted in females of more rapidly growing species. Anat. Rec., **62**:223–45.

———. 1936. Sex reversal in parabiotic *A. punctatum* of various local races. Amer. Jour. Anat., **59**:347–63.

KOZELKA, A. W., and GALLAGHER, T. F. 1934. Effect of male hormone extracts, theelin, and theelol on the chick embryo. Proc. Soc. Exper. Biol. and Med., **31**:1143.

LILLIE, FRANK R. 1917. The free-martin: a study of the action of sex hormones in the foetal life of cattle. Jour. Exper. Zoöl., **23**:371–452.

MCCRADY, EDWARD, JR. 1938. The embryology of the opossum. Amer. Anat. Memoirs, No. 16. Wistar Institute.

———. 1940. The development and fate of the urinogenital sinus in the opossum, *Didelphys virginiana.* Jour. Morph., **66**:131–54.

Moore, Carl R. 1939. Modification of sexual development in the opossum by sex hormones. Proc. Soc. Exper. Biol. and Med., **40**:544–46.

———. 1940. Effects of testosterone propionate on embryonic ducts in the rat. Arch. intern. phar. et therap. (in press; fate uncertain due to German occupation of Belgium).

Moore, Carl R., and Bodian, David. 1940. Opossum pouch young as experimental material. Anat. Rec., **76**:319–27.

Moore, Carl R.; Lamar, Jule K.; and Beck, Naomi. 1938. Cutaneous absorption of sex hormones. Jour. Amer. Med. Assn., **111**:11–14.

Plagge, J. C., and Lamar, J. K. 1939. Natural occurrence of imperforate vagina in the adult female albino rat. Proc. Soc. Exper. Biol and Med., **42**:52–53.

Price, Dorothy. 1936. Normal development of the prostate and seminal vesicles of the rat with a study of experimental post-natal modifications. Amer. Jour. Anat., **60**:79–127.

———. 1939. Normal development and regression of the prostate gland of the female rat. Proc. Soc. Exper. Biol. and Med., **41**:580–83.

1941. Rat prostate and seminal vesicle grafts in relation to the age and sex of the host. Physiol. Zoöl., **14** (April [in press]).

Puckett, W. O. 1940. Some effects of crystalline sex hormones on the differentiation of the gonads of an undifferentiated race of *Rana catesbiana* tadpoles Jour. Exper. Zool., **84**:39–51.

Raynaud, A. 1938. Intersexualité obtenue expérimentalement chez la souris femelle par action hormonale. Bull. biol., **72**:297–354.

Risley, Paul L. 1937. A preliminary study of sex development in turtle embryos following administration of testosterone. Anat Rec., **70**, Suppl No. 1, 103.

Turner, C. D. 1939. The modification of sexual differentiation in genetic female mice by the prenatal administration of testosterone propionate Jour. Morph , **65**:353–81.

———. 1940. The influence of testosterone propionate upon sexual differentiation in genetic female mice; post-natal androgens alone and in combination with prenatal treatments Jour. Exper. Zoöl., **83**:1–31.

Wiesner, B. P. 1935. The post-natal development of the genital organs in the albino rat. Jour. Obstet. and Gynec of British Empire, **41**:867; **42**:8.

Willier, B. H. 1921. Structures and homologies of free-martin gonads. Jour. Exper. Zool., **33**:63–127.

———. 1939. The embryonic development of sex. In Sex and internal secretions, chap. iii. Baltimore: Williams & Wilkins.

Willier, B. H.; Gallagher, T. F., and Koch, F. C. 1937. The modification of sex development in the chick embryo by male and female sex hormones. Physiol. Zool., **10**:101–22.

Willier, B. H.; Rawles, Mary E ; and Koch, F. C. 1938 Biological differences in the action of synthetic male hormones on the differentiation of sex in the chick embryo. Proc. Natl. Acad. Sci., **24**: 176–82.

Wislocki, Geo. B. 1939. Observations on twinning in marmosets. Amer. Jour. Anat., **64**:445–83.

Witschi, Emil. 1927. Sex reversal in parabiotic twins of the American wood-frog. Biol. Bull., **52**:137–46.

———. 1929. Studies on sex differentiation and sex determination in amphibians. II. Sex reversal in female tadpoles of *Rana sylvatica* following the application of high temperature. Jour. Exper. Zoöl., **52**:267–91.

———. 1937. Studies on sex differentiation and sex determination in amphibians. IX Quantitative relationships in the induction of sex differentiation, and the problem of sex reversal in parabiotic salamanders. *Ibid.*, **75**:313–73.

———. 1939. Modification of the development of sex in lower vertebrates and in mammals. In Sex and internal secretions, chap. iv. Baltimore: Williams & Wilkins.

Wolfe, E., and Ginglinger, A. 1935. Sur la transformation des poulets mâles en intersexués par injection d'hormone femelle (folliculine) aux embryons. Arch. d'anat., d'hist., et d'embryol., **20**: 219–78.

Womack, E. B., and Koch, F. C. 1930. Biochemical studies on the yield of testicular hormone from tissues and body fluids and on factors affecting the comb growth response in the brown leghorn capon. Proc. 2d Intl. Cong. for Sex Research (London), p. 329.

PLATE I

FIG. 1.—Photograph of open marsupial pouch showing young on day 3 attached to nipples (*see low center*). About 1½×.

FIG. 2.—Opossum pouch young on day 35. Note nipple attachment and vacated nipple from which a treated young had just been removed. Note prominence of external genitalia from androgenic treatments. About 1½×.

PLATE II

FIG. 3.—Drawing of normal adult male reproductive system; tunica removed from testis on right.

PLATE III

FIG. 4.—Drawing of adult normal female reproductive system.

PLATE IV

FIG. 5.—Drawing of 31-day normal female reproductive tract (ser. 18). *A*, adrenal; *Bl*, bladder; *K*, kidney; *LVC*, lateral vaginal canal; *OV*, ovary; *Ovd*, oviduct; *Ut*, uterus.

FIG. 6.—Drawing of 31-day treated female reproductive tract (ser. 64B). Testosterone-propionate treatments on days 10–30. Drawn to scale of Fig. 5. *A*, adrenal; *Bl*, bladder; *K*, kidney; *LVC*, lateral vaginal canal; *Ut*, uterus; *WD*, Wolffian duct.

PLATE V

FIGS. 7–16.—Photomicrographs from opossum serial sections (same magnification for all figures). Original, 30×.

C, cervix
LVC, lateral vaginal canal
MD, Müllerian duct
O, ostium
OV, ovary
UGS, urogenital sinus
UT, cavity of uterus
UTG, uterine glands
WD, Wolffian duct

FIG. 7.—Normal female, 31 days old (ser. 18; see Fig. 5), showing cervical canal in genital cord and sections through uterus. Gut above.

FIG. 8.—Androgen-treated female, 31 days old (64B; see Fig. 6), showing enormous development of the Müllerian duct into a uterine cavity, precociously differentiated uterine glands, ostium, and ovary; Wolffian ducts are visible.

FIG. 9.—Androgen-treated male, 31 days old (64A; same treatment as female in Fig. 8). Two sections of the Wolffian duct reveal its relative unresponsiveness; Müllerian duct, though preserved, exhibits minimal response (cf. Fig. 8). Gut above; urogenital sinus (*center*) shows precociously developed prostate (cf. Fig. 10).

FIG. 10.—Normal male, 32 days old (34B), showing early prostate-cord outgrowth from urogenital-sinus epithelium.

FIG. 11.—Androgen-treated male, 30 days old (101A). Prostatic outgrowths from urogenital-sinus epithelium greatly exceed the normal (cf. Fig. 10).

FIG. 12.—Androgen treated female, 30 days old (101B). Prostatic induction and precocious development (compare with normal male, Fig. 10).

FIG. 13.—Estrogen-treated male, 31 days old (39A). Urogenital-sinus epithelium shows tremendous hyperplasia and metaplasia; proliferation, instead of peripheral for prostate formation, has been central with sloughing of cells into the lumen of the urogenital sinus.

1

2

PLATE II

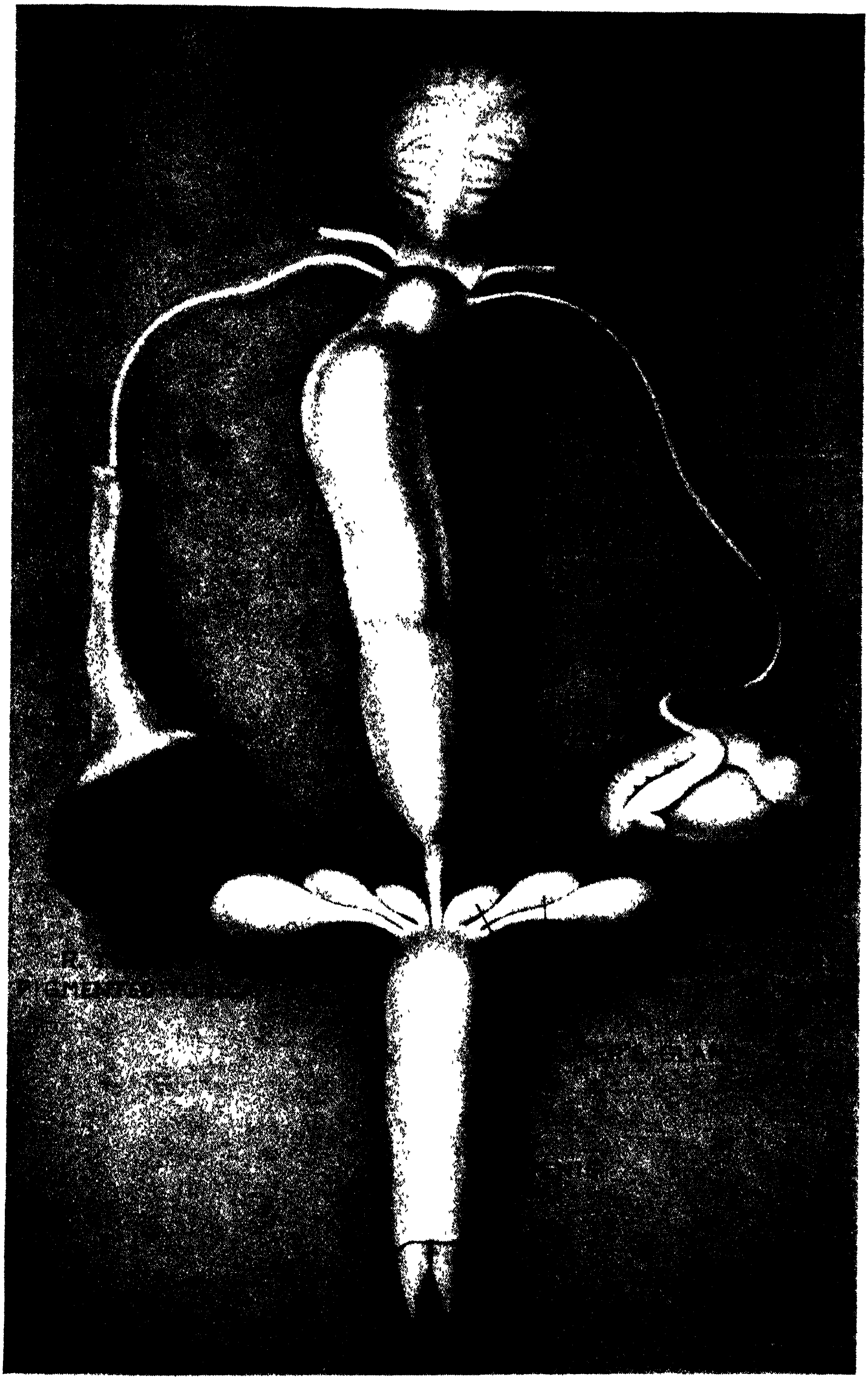


Fig. 3

PLATE III

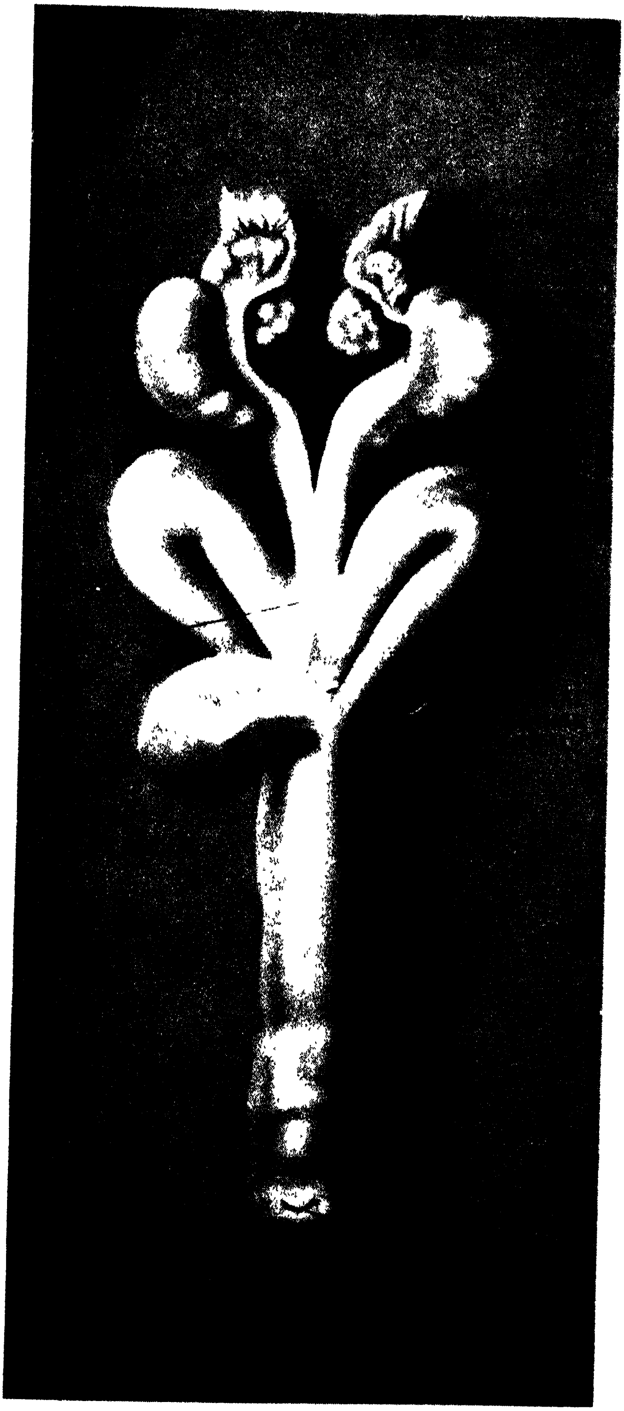


Fig. 4

PLATE IV

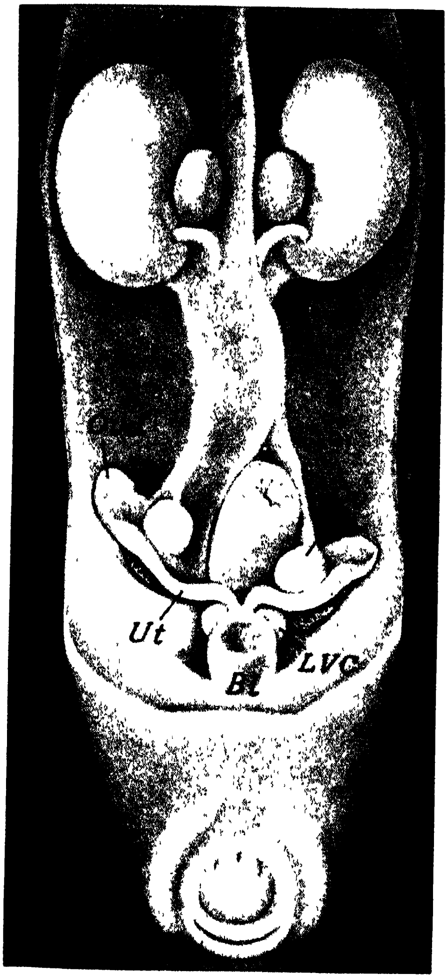


FIG. 5

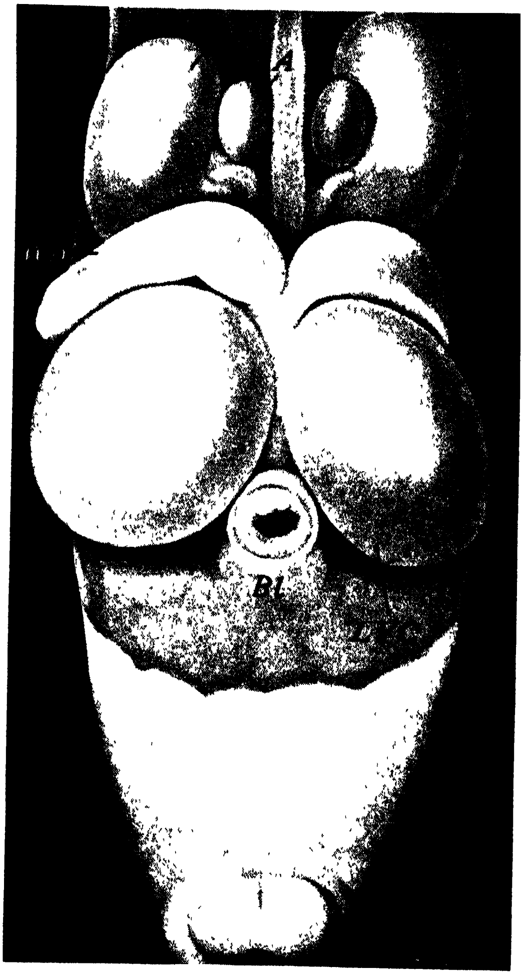
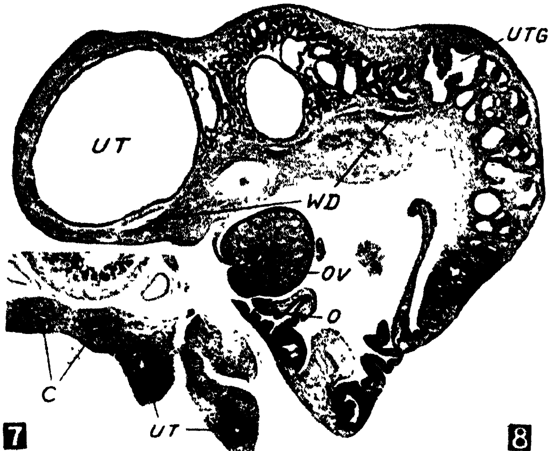


FIG. 6

PLATE V



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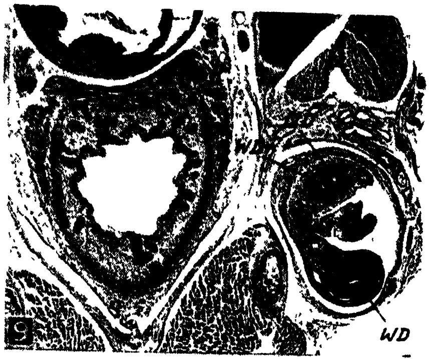

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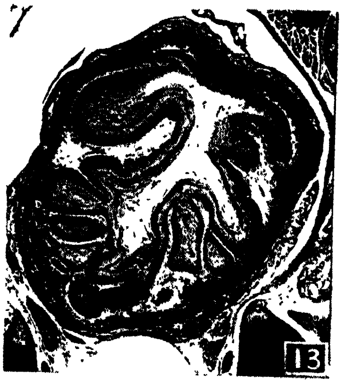

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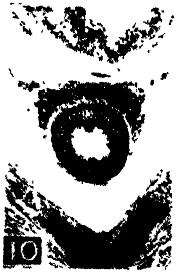

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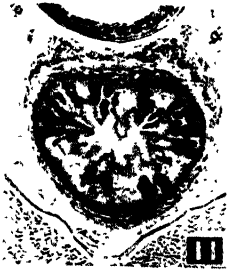

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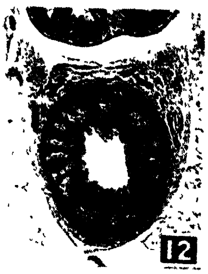

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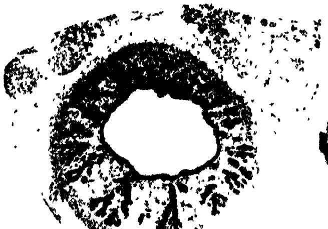

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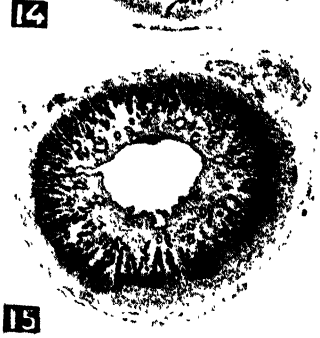

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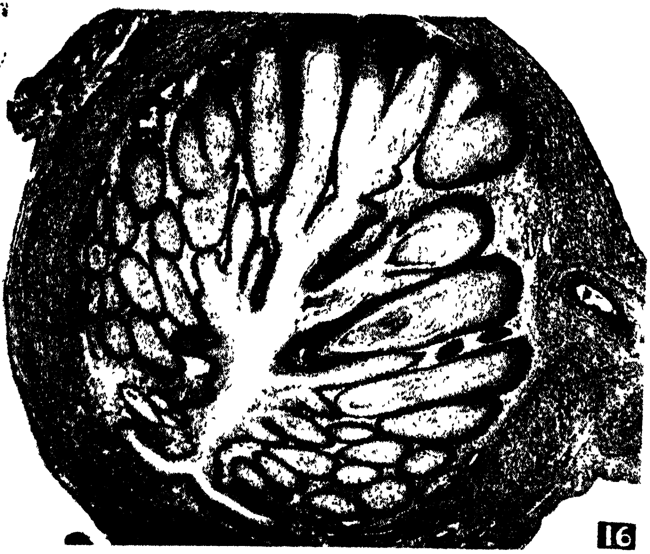

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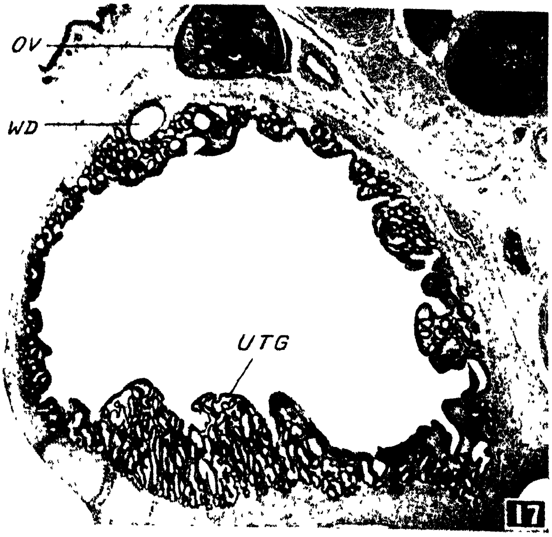
PLATE VI



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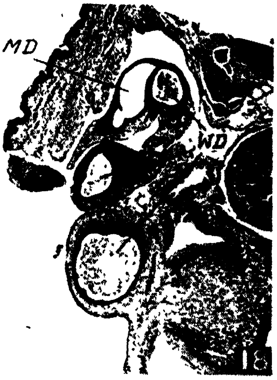

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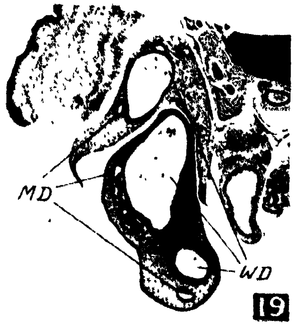

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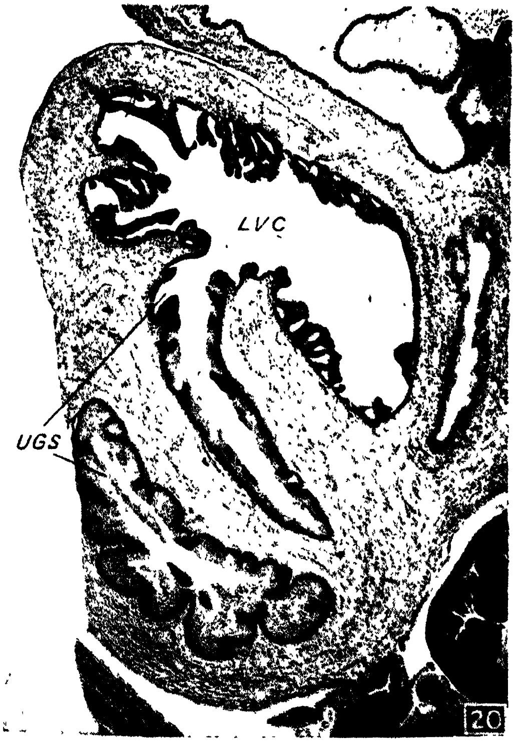

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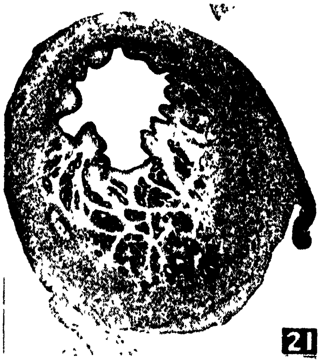

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FIG. 14.—Untreated male, 100 days old (88), showing prostate gland as solid cords of cells, without canalization.

FIG. 15.—Androgen-treated female, 95 days old (118); treatments on days 3–44; no treatment on days 45–95. Prostate-gland induction, and differentiation much beyond normal male of age (cf. Fig. 14.).

FIG. 16.—Estrogen-treated male, 83 days old (93); treatments on days 70–82. The previously formed prostatic outgrowths and urogenital-sinus epithelium have undergone tremendous hyperplasia and metaplasia.

PLATE VI

FIGS. 17–22.—Photomicrographs from opossum serial sections (magnifications same in Pls. V and VI). Original, 30×. (For labels see Pl. V.)

FIG. 17.—Estrogen-treated female, 46 days old (ser. 115). For increased uterine diameter compare with Fig. 7; for precocious uterine-gland development compare with 100-day normal female (Fig. 22). Ovary appears normal. Wolffian duct slightly posterior becomes size of uterus in this section.

FIG. 18.—Estrogen-treated male, 23 days old (55A).

FIG. 19.—Estrogen-treated female, 23 days old (55B). For greater Wolffian-duct response to estrogens than to androgens compare Figs. 18 and 19 with the androgen-treated male of Fig. 9.

FIG. 20.—Estrogen-treated female, 46 days old (115; same young as Fig. 17), showing transition of glandular epithelium of lateral vaginal canal into upper portion of urogenital sinus.

FIG. 21.—Androgen-treated female, 95 days old (117). This androgen-stimulated uterus, with its glandular differentiation, should be compared with the normal 100-day uterus of Fig. 22.

FIG. 22.—Normal female 100 days old, to show cross-section of the uterus; no glandular differentiation.

AN EXPERIMENTAL STUDY ON THE ORIGIN OF MELANOPHORES IN BIRDS

(Four figures and three plates)

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THE origin of pigmentation in the integumentary structures of vertebrates has been widely discussed from the time of the earliest investigations. The first authors were impressed by the occurrence in the epidermis and dermis of very characteristic starlike cells carrying melanin granules. Two questions arose: Do these cells belong to the mesoderm or to the ectoderm? Is the melanin pigment formed only in these cells, or do other cells have the capacity to produce it? The answers which have been given to these questions up to the present time can be classified into three groups. As early as 1860 Kölliker described these pigment cells in *Protopterus* and concluded that they were derived from mesenchymal cells which migrated into the epidermis. This view was confirmed by Kerbert (1877) for reptiles and birds, Riehl (1884) for man and birds, and Ehrmann (1885) for man, various mammals, and birds. Haecker (1890) studied pigmentation in bird feathers and came to the conclusion that pigmentation of feathers was caused by migrating pigment cells of mesenchymal origin.

However, such investigators as Mertsching (1889), Klee (1886), Strong (1902), and Winkler (1910) reached quite a different interpretation. In their opinion the pigment was formed within the epidermal cells of amphibians, birds, and mammals. The pigment cells in the mesenchyme were believed to be derivatives of epithelial cells as Post (1894) had pointed out. Still other investigators (Jarisch, 1892; Caspary, 1891; Lloyd-Jones, 1915; Greite, 1934) tried to combine both viewpoints. While they recognized migrating pigment cells, they attributed to the epithelial cells also the capacity to produce melanin.

It is evident that the source of the pigment cells cannot be decided by histological methods alone, since in their early stages they cannot be distinguished from other cells. Experiments in amphibians lead to a strikingly different interpretation. Harrison (1910), Weidenreich (1912), and Holtfreter (1933) obtained evidence which showed that the melanophores of amphibians are not derived either from the epidermis or from the mesenchyme but from the neural crest. Du Shane (1934, 1935) was able to demonstrate this in *Ambystoma* by xenoplastic transplantations of neural crest and control tissues. The appearance of donor melanophores in hosts could be checked, since the different species have specific pigment cells.

In the chick the problem was attacked by Dorris (1936, 1938, 1939). From studies in tissue culture and grafting to the leg-bud stage she showed that neural crest produced melanophores, but skin and mesoderm failed to do so. Willier and Rawles (1940) have found that grafts of wing or leg skin and mesoderm to the wing bud produce melanophores only for donors beyond a certain age. Watterson (1938) obtained similar results for pure mesoderm. Eastlick (1938 and 1939*b*) has shown that the pigment-producing agent must originate in the neural-tube region and spread laterally in later development. Leg buds of 70-hour chick embryos of pigmented breeds, cut at the edge of the neural tube and transplanted into the coelom of White Leghorn embryos of the same age, developed pigmented down feathers. If severed at the level of the intermediate cell mass, however, unpigmented down resulted. Furthermore, in older embryos the more distal parts of the limbs, if grafted into the coelom, developed pigment.

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[1] Research fellow in embryology. I wish to thank Professor B. H. Willier for initiating this problem and for his interest during the course of the work.

The present investigation was designed to obtain further evidence from experimental and histological studies on the extra-epidermal origin of the melanophores in birds and their derivation from the neural crest. In addition, the origin of the retinal and chorioid pigment of the eye was analyzed.

MATERIAL AND METHODS

To test the potency of melanin formation various tissues of bird embryos of pigmented breeds and of different ages and levels were transplanted to host embryos. Small isolated pieces were grafted (1) into the wing bud of 70-hour White Leghorn embryos; (2) into the coelom of 60–70-hour White Leghorn embryos; (3) onto the chorio-allantois of White Leghorn, Barred Plymouth Rock, and F_1 hybrid (Barred Rock ♀ × Rhode Island Red ♂). The technique used for the first series of experiments was described by Willier, Rawles, and Hadorn (1937) and by Rawles (1939); for the second by Hamburger (1938); for the third by Rous and Murphy (1911), and for embryological research by Willier (1024).

The donors were usually Barred Plymouth Rock; but various pigmented breeds of fowl including Brown Leghorn, New Hampshire Red, F_1 hybrid (Barred Rock ♀ × Rhode Island Red ♂) were also used. In addition, Mongolian and Formosan Pheasants, robin (*Turdus migratorius*), and Black Fantail Pigeon served as donors.

Selected grafts were fixed in Bouin, sectioned at 7 and 12 μ, and stained with Delafield's haemotoxylin and eosin. Such preparations demonstrated clearly the absence or presence of melanin. Further details will be given in the appropriate sections.

EXPERIMENTS

I. ORIGIN OF MELANOPHORES OUTSIDE OF EPIDERMIS AND MESENCHYME

The experiments of Dorris (1936, 1938, 1939) and Eastlick (1938 and 1939*b*) mentioned above show a high degree of independence of the melanin pigmentation and epidermal structures. This is even more strikingly demonstrated by xenoplastic grafts of embryonic robin tissue into the wing bud of White Leghorn embryos (Rawles, 1939) where White Leghorn feathers carrying specific robin pigment granules were obtained.

To obtain further evidence for this independence and to trace the lateral spreading of the pigment-forming capacity, pieces of embryos of varying age were isolated and transplanted onto the chorio-allantois of 8-day hosts or into the coelom of White Leghorn embryos of 60–70 hours. Isolates of about 1 somite in length were cut at different longitudinal levels and at varying lateral distances from the neural tube. The cut, for instance, ran between the neural tube and the somite through the intermediate cell mass or through the middle of the limb bud, etc.

The chorio-allantoic grafts were examined with a dissecting microscope, and the presence or absence of melanophores ascertained. Certain grafts were sectioned and studied for the presence of pigment cells. The hosts with coelomic grafts were killed before hatching and dissected. It was found that the transplants which developed in the body cavity were attached to the peritoneum of the body wall, to the mesenteries, or to both. Very often the connection was only a strand of tissue containing a blood vessel. The skin ectoderm of the implant developed in the majority of cases into a sac with the

TABLE 1*

ORIGIN OF MELANOPHORES OUTSIDE OF EPIDERMIS AND MESENCHYME

Case No.	Donor	Level	Isolate	Host	Melanophore Production	Remarks
			A. Fowls			
16-12.	BR 3 s.	node	node+ heart	WL ch-a.	+	
16-2.	BR 3 s.	3 s.	neural fold	WL ch-a.	+	contracting heart muscles
48-13	BR 11 s.	8/9 s.	nt-som	WL coelom	—	White down
6-2.	BR 18 s.	16 s.	nt-som	WL coelom	—	White down
6-3.	BR 18 s.	16 s.	nt-som	WL coelom	—	White down
15-7.	BR 18 s.		forebrain floor	BR ch-a.	+	
11-7....	Red 19 s.	node	node	WL coelom	+	Red down
39-2.	BR 23 s.	10 s.	nt-som	WL ch-a.	—	
13-3.	BR 25 s.	16 s.	nt-som	WL coelom	—	White down
39-7..	BR 25 s.	22 s.	nt-som	BR ch-a.	—	
22-7.	BR 27 s.	16 s.	nt-som	WL ch-a.	—	
12-15	BR 29 s.	16 s.	nt-som	WL ch-a.	+	
30-16.	F_1 30 s.	16 s.	nt-som	WL coelom	+	Black down on abnormal wing
35-2..	BR 30 s.	19 s.	nt-som	WL ch-a.	+	
44-5.	BR 30 s.	22 s.	nt-som	BR ch-a.	—	
44-11.	BR 30 s	bseg.	nt-som	BR ch-a.	—	
44-14	BR 31 s.	22 s.	imt	BR ch-a.	—	
52-4...	BL 34 s.	25 s.	imt	F_1 coelom	—	F_1 pigment invaded graft†
41-4.	BR 34 s.	33 s.	nt-som	BR ch-a.	—	
9-7.	BR 37 s.	16 s.	imt	WL ch-a.	—	Feather germs without melanophores
9-8.	BR 37 s.	16 s.	distal half s.	WL ch-a.	—	
7-2. .	BR 37 s.	head	skin	WL coelom	+	Pigmented skin and down
12-18	BR 47 s.	26 s.	tip leg bud	Red ch-a.	—	
9-12.	BR 47 s.	16 s.	ventral skin	WL ch-a.	—	
			B. Other Birds			
55-5....	RP 16 s.	10 s.	nt-som	WL coelom	+	Skin and down with melanophores
55-6. .	RP 16 s.	bseg.	nt-som	WL coelom	—	White down
57-5. .	Robin 7s.	bseg.	nt-som	BL coelom	—	Few BL melanophores invaded graft†
60-8. .	BPg 2 days	15 s.	nt-som	WL coelom	—	White down

* BR = Barred Plymouth Rock; WL = White Leghorn; BL = Brown Leghorn; Red = New Hampshire Red; F_1 = Hybrid (Barred Rock ♀ × Rhode Island Red ♂); RP = Ringnecked Pheasant; BPg = Black Fantail Pigeon; ch-a. = chorio-allantois; nt-som = cut between neural tube and somite or derivatives; s. = somite; bseg. = behind segmental plate; imt = cut through intermediate cell mass; + = presence of a structure; — = absence of a structure.

† The different breeds of fowl and species of birds have specific melanin granules (Danforth, 1937; Rawles, 1939).

down feathers directed inward (see Pl. I, Fig. 2). Less often the feathers grew directly into the body cavity (see Fig. 1). The results of the transplantations are given in Table 1. Comparing the different transplants, one can see that the pigment-producing agent spreads laterally from the neural tube, first in the anterior region and gradually in more posterior levels.

An analysis of these data leads to the following conclusions:

1. No pigment is formed in either epidermal or mesenchymal cells. The pigment-producing agent therefore comes from outside of the mesenchyme and skin. In very young donors only the neural-tube region gives pigment. In older embryos more and more lateral tissues can produce pigment cells. This shows that some factor necessary for pigment formation migrates laterally, first in the more anterior levels and later in the posterior parts of the embryo.
2. The source of the pigment lies in the region of the neural tube. At every age and level the neural tube gives pigment. At the wing-bud level the pigment-producing agent invades the tissues adjacent to the neural tube between the 27-somite and the 29-somite stages (e.g., cases 22-7; 12-15 in Table 1).
3. No difference in melanophore production is found in chorio-allantoic grafts on the White Leghorn or Barred Rock and F_1 hybrid host embryos. This excludes the presence of any differential substance in the blood stream necessary for pigment production.

II. DEVELOPMENT OF THE NEURAL CREST AND THE APPEARANCE OF MELANOPHORES IN GRAFTS

Du Shane has shown that melanophores are derived from the neural crest in amphibians. Considering the results of the preceding experiments, we immediately think of the neural crest as the source of the pigment cells, since neural-crest cells are known to have the capacity to migrate in the body. The problem now is to find out whether there is any correlation between the development of the neural crest and the observed spreading of the pigment-forming agent.

In 1928 Holmdahl showed in a very extensive and thorough study of the neural crest in birds (and mammals) that in the chick the neural crest arises first in the midbrain region of a 6-somite embryo. It never forms a continuous ridge throughout the embryo but develops as a wave proceeding caudally. The typical development at any level may be described as follows. The tissue of the dorsal part of the closed neural tube, covered by a thin layer of epidermis, becomes loose and irregular. Cells from this loose tissue form an irregular swelling on both dorsolateral edges of the neural tube. Later these cells leave the neural tube, migrating in strands both ventrally along the lateral wall of the neural tube and laterally between the dermatome and the skin ectoderm. These strands often form a characteristic fork at the edge of the dermatome. Are these cells migrating out from the neural crest the precursors of the melanophores?

The following experiments link the neural-crest cells with the appearance of melanophores. Chick embryos of about 20–30 somites show the neural crest in all stages of its development. In an anterior level the neural-crest cells have left the neural tube and have wandered out along its lateral walls and dorsal to the dermatome (Fig. 6). Posteriorly, we find either the neural-crest cells in a ridge on both dorsolateral edges of the neural tube (Fig. 5) or no morphological indication of a neural crest at all.

It is therefore possible to cut lateral pieces at different levels along the edge of the neural tube so that the anterior ones include neural-crest cells and the posterior ones do not. With a sharp iridectomy knife such pieces were cut away from the neural tube on the right side of the donor. The corresponding left side was fixed and sectioned (Fig. 15).

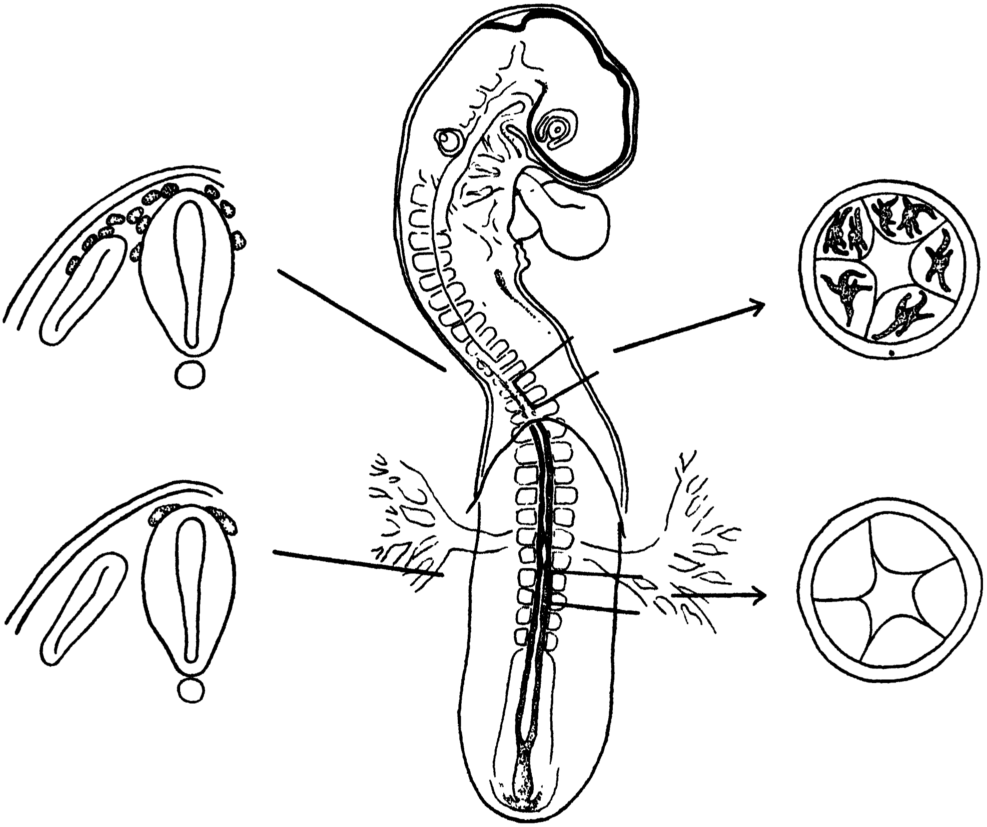

FIG. 15.—Diagram showing the correlation between the development of the neural crest and pigment formation in chorio-allantoic grafts. The right side shows the manner of isolation and whether or not melanophores are formed. On the left side the stages of neural-crest development in levels corresponding to the isolates are seen.

TABLE 2*

CORRELATION BETWEEN THE DEVELOPMENT OF THE NEURAL CREST AND THE APPEARANCE OF MELANOPHORES IN GRAFTS

Case No	Donor	Level	Neural-Crest Stage	Host	Melanophore Production
39-2	BR 23 s.	10 s.	Crest	WL ch-a.	—
44-1	BR 30 s.	bseg.	Not formed	BR ch-a.	—
35-10	BR 30 s.	26 s.	Migrating cells	WL ch-a.	+
44-17	BR 31 s.	bseg.	Not formed	BR ch-a.	—
34-8	BR 31 s.	25 s.	Migrating cells	BR ch-a.	+

* For abbreviations see note to Table 1.

Thus the appearance of melanophores in the graft can be compared with the outgrowth of the neural crest. The results of these experiments are summarized in Table 2.

The data given in Table 2 show that there is a close relationship between the stage reached in the development of the neural crest and the appearance of melanophores in grafts of pieces lateral to the neural tube. In all cases where no pigment was found in the ectodermal and mesodermal structures of the graft histological examination of the intact side of the donor showed that the neural-crest cells formed a ridge dorsolateral to the neural tube. No cells had migrated from the ridges. The grafts therefore contained no crest cells (Fig. 5). On the other hand, when the cells had left the neural crest and could be seen over the dermatome (Fig. 6), the grafted piece cut at the edge of the neural tube contained neural-crest cells. In these cases the transplant on the chorio-allantois contained an abundance of melanophores. Melanophores, therefore, are produced only by grafts containing neural-crest cells.

III. THE NEURAL CREST AS THE SOLE SOURCE OF THE MELANOPHORES

Although all the evidence at hand points strongly to the neural crest as the source of the melanophores, the possibility still remains that they may arise from some other region of the neural tube. In 1939 Jones presented evidence that sheath cells—another derivative of the neural crest—may take their origin also from the ventral floor of the neural tube.

It was not found possible to isolate neural crest of the chick embryo entirely free from neural-tube and skin-ectodermal cells. Invariably, some of these cells are included. Consequently, the development of melanophores in such grafts does not prove their sole origin from the neural crest, contrary to what Dorris (1936, 1938, 1939) has maintained.

It is possible, however, to cut pieces of the neural tube (1–2 somites in length) into dorsal and ventral parts and to test their pigment-forming capacities. If such pieces are taken at an age or level at which the neural-crest cells have not yet migrated along the lateral walls of the neural tube, the ventral half would be free of them. The dorsal part contains either the presumptive or the morphologically observable neural crest (Fig. 16).

These dorsal and ventral parts of neural tube were first grafted into the wing bud of 72-hour White Leghorn embryos (Table 3, ser. 26, 27, 33). But, as the graft is generally absorbed in the wing bud, it was considered more suitable to transplant the pieces into the coelom so that a distinct graft could be obtained in all cases. A slit was made anterior to the right-leg bud of 60–70-hour White Leghorn embryos, and the implant pushed into the coelom. This site was found to provide the best conditions foı growth. Table 3 gives the data of these experiments.

The grafts in the wing bud show that the ventral half of the neural tube fails to produce any pigment, if it does not contain neural-crest cells (see Table 3, cases 27-5; 27-7; 27-3; 26-8). When the neural-crest cells have migrated ventrally, the grafted ventral portion will produce melanophores (see cases 26-6; 33-4; 33-6). All dorsal grafts produced melanophores in the skin and feathers of the right wing of the host.

Still more convincing are the transplants which develop in the coelom of White Leghorn embryos. The ventral and dorsal halves of neural tube formed distinct grafts, attached either to the coelomic epithelium of the body wall adjacent to the mesonephros (Fig. 7) or to the mesenteries (Fig. 8).

The ventral half, containing no neural-crest cells, differentiates into a small body

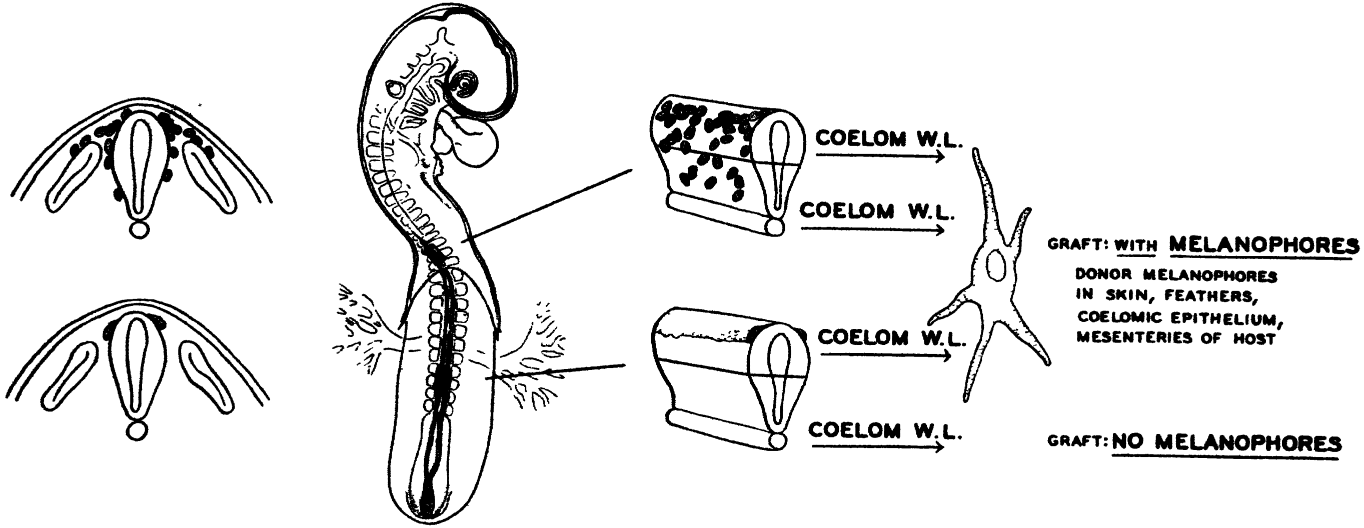

FIG. 16.—Diagram showing the isolation of dorsal and ventral halves of the neural tube at different levels of the donor embryo, together with the stage of development of the neural crest. Whether or not these isolates when transplanted to the coelom of the White Leghorn give rise to melanophores is shown at the right.

TABLE 3

THE NEURAL CREST AS SOLE SOURCE OF THE MELANOPHORES

A. FOWLS

Case No.	Donor	Level	Neural-Crest Stage*	Implant	Host	Melanophore Production
27-5	BR 9 s.	9 s.	Not formed	Ventral half†	WL wing bud	—
27-6	BR 9 s.	5 s.	Not formed	Dorsal half	WL wing bud	+
27-7				Ventral half	WL wing bud	—
26-5	BR 11 s.	5 s.	Migrating cells	Dorsal half	WL wing bud	+
26-6				Ventral half	WL wing bud	+
26-7	BR 11 s.	9 s.	Crest	Dorsal half	WL wing bud	+
26-8				Ventral half	WL wing bud	—
33-7	BR 13 s.	3 s.	Migrating cells	Dorsal half	WL coelom	+
33-8				Ventral half	WL coelom	+
47-2	BR 18 s.	17 s.	Crest	Ventral half	WL coelom	—
47-3	BR 18 s	14 s.	Crest	Ventral half	WL coelom	—
65-5	BR 19 s.	bseg	Not formed	Ventral half	WL coelom	—
46-4	BR 22 s.	22 s.	Crest	Ventral half	WL coelom	—
30-2	F_1 23 s.	7 s	Migrating cells	Dorsal half	WL coelom	+
30-3				Ventral half	WL coelom	+
40-8	F_1 30 s.	11 s.	Migrating cells	Ventral half	WL coelom	+
40-4	F_1 33 s.	10 s.	Migrating cells	Ventral half	WL coelom	+
27-2	BR 35 s.	25 s.	Crest	Dorsal half	WL coelom	+
27-3				Ventral half	WL coelom	—
42-8	BR 35 s.	30 s	Crest	Ventral half	WL coelom	—
42-9	BR 35 s.	28 s.	Crest	Dorsal half	WL coelom	+
42-10				Ventral half	WL coelom	—
33-4	BR 37 s.	35 s.	Migrating cells	Ventral half	WL wing bud	+
33-5	BR 37 s.	30 s.	Migrating cells	Dorsal half	WL wing bud	+
33-6				Ventral half	WL wing bud	+
9 grafts from dorsal halves from various levels and ages					WL wing bud and WL coelom	+

B. OTHER BIRDS

Case No.	Donor	Level	Implant	Host	Melanophore Production	Remarks
56-5	MP‡ 26 s.	24 s.	Ventral half	WL coelom	—	
56-6.	MP 26 s.	20 s.	Ventral half	WL coelom	—	
56-11	FP 39 s.	....	Neural tube	WL leg bud	+	Leg with pheasant-colored down
57-7	Robin 7 s.	bseg.	Ventral half	BL coelom	—	No robin melanophores
57-8	Robin 7 s.	7 s.	Ventral half	WL coelom	—	
57-9.	Robin 7 s.	4 s.	Ventral half	WL coelom	—	
60-3.	Pigeon 2 days	19 s.	Ventral half	WL coelom	—	

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* Neural-crest stages based on series of normal development For birds other than fowl unknown For other abbreviations see Table 1.

† Dorsal half = dorsal half of neural tube, ventral half = ventral half of neural tube.

‡ MP = Mongolian Pheasant, FP = Formosa Pheasant.

without melanophores, and no donor melanophores are found in the host tissues (Table 3, cases 47-2; 47-3; 46-4; 42-8; 42-10; see Fig. 7). Ventral halves containing neural-crest cells (Fig. 9) and dorsal halves produce grafts of similar shape from which large numbers of melanophores have migrated into the peritoneum, mesenteries, skin, and feathers of the host. In some cases the graft itself was entirely free of pigment cells, all of which had apparently migrated into the host (Fig. 9).

Both the dorsal and the ventral halves of the same piece of neural tube developed in three wing-bud grafts and in one in the coelom. The dorsal produced melanophores, the ventral did not (Table 3, cases 27-6 and 7; 27-2 and 3; 42-9 and 10).

One graft of a dorsal half and one of a ventral portion were fixed in Bouin, sectioned (7 μ), and stained with haematoxylin-eosin. The dorsal half forms ganglion cells scattered through the graft, nerve cells often being arranged in short tubes and with nerve fibers and melanophores distributed peripherally (Fig. 12). The ventral part with the notochord differentiates into cartilage, nerve cells, ganglion cells, nerve fibers, but no melanophores (Fig. 13).

Ventral halves of neural tube from the region of the most posterior somites formed melanophores in four out of nine grafts. Five out of six from behind the somite region gave pigment. These results may have the following explanations. The neural tube of the more posterior levels is very thin and difficult to cut. The production of pigment by the ventral part may be due to an imperfect separation of the tissues. The following seems more probable. Since the whole neural plate has the potency to form melanophores (see p. 63), the ventral part of the neural tube must lose this potency during development. The above results may indicate that the ventral part has not yet lost this potency.

From these experiments it is concluded that pieces of the neural tube produce pigment only if they contain presumptive neural crest or recognizable crest-cells. The neural crest is the sole source of the melanophores in all the birds tested, i.e., in fowl, pheasant, robin, and pigeon.

IV. THE ORIGIN OF THE EYE PIGMENT

Kölliker in 1887 called attention to the fact that the pigment in the outer layer of the retina originates independently of branched pigment cells. This has often been used as an argument against the dominating role of the branched melanophores in the pigmentation of vertebrates. The eye of the chick contains pigment not only in the retina but also in the chorioidea and iris. The pigmentation of the retina is due to a layer of polygonal cells which produce pigment granules *in situ*, beginning during the fourth day of incubation. The pigment in the mesodermal coat of the eye vesicle (chorioidea and part of iris) is restricted to branched pigment cells, which produce pigment first at the end of the seventh day of incubation. These cells are similar to the melanophores found in the skin and mesenchyme.

The origin of the pigment in the chorioidea has been much discussed. Kölliker believed that mesenchymal cells differentiating into pigment cells invaded the chorioidea. Lewis (1903) contended that the wandering pigment cells are derived from the pigmented epithelium of the retina. Szily (1911) thought it possible that cells of the chorioidea differentiate into pigment cells. It was Bisceglie (1932) who showed that in tissue culture isolated chorioidea gives rise to mesenchyme cells and melanophores and that isolated retina produces a layer of polygonal cells containing melanin granules but never branched pigment cells.

Further evidence for the difference between the retinal pigment cells and the melanophores is provided by the character of the eye pigmentation in the White Leghorn. In this breed Dorris (1936, 1938) found that explants from embryos containing neural crest formed melanophores in tissue culture. In a special study of the normal White Leghorn embryo I have found a number of melanophores also in the skin and beneath the coelomic epithelium after the seventh day of incubation. At the time of hatching, the skin melanophores accumulate at the base of the down feathers, leaving the epidermis almost colorless as in most darkly pigmented breeds. The White Leghorn melanophores contain small dark granules, but only few of these cells are filled with them. Colorless granules are more abundant. All these melanophores migrate into the feather germs. The fact that the number containing black granules is small is probably one of the chief factors causing the "whiteness" of White Leghorn feathers. In other words, a small quantity of pigment is distributed over a wide area.

TABLE 4*

THE DIFFERENCE IN PLACE OF ORIGIN OF RETINAL AND CHORIOID PIGMENT

Case No	Donor	Implant	Neural Crest† Stages	Host	Retinal Pigment	Melanophore Production
48-2	BR 6 s.	Left optic vesicle	Crest mid-brain level	WL coelom	+	–
48-4	BR 6 s	Left optic vesicle plus midbrain	Migrating cells in midbrain	WL coelom	+	+
48-7	BR 8 s.	Distal part of eye vesicle	Migrating cells in midbrain	WL coelom	+	–
48-12	BR 11 s.	Right forebrain with optic vesicle	Crest in forebrain level	WL coelom	+	+
48-0	BR 12 s.	Distal part of eye vesicle	Beginning cell migration at forebrain level	WL coelom	+	–
48-10	BR 12 s	Distal part of eye vesicle	Beginning cell migration at forebrain level	WL coelom	+	–

* For abbreviations see previous tables.

† Neural crest stages based on normal development.

The retinal pigment in the White Leghorn embryos is as dark and abundant as in a dark breed. But the chorioidea contains a small number of branched pigment cells with as few black granules as in those found in the skin. This striking difference in the pigment formation is further evidence for the independent origin of retinal and chorioid pigment.

To determine whether retinal cells may give rise to branched pigment cells, pieces of the eye cup of a 72-hour White Leghorn embryo were grafted into the wing bud of a 70-hour White Leghorn host. At hatching the graft had formed a small rounded clump of retinal epithelium at the base of the host wing. No dark cells had migrated out into

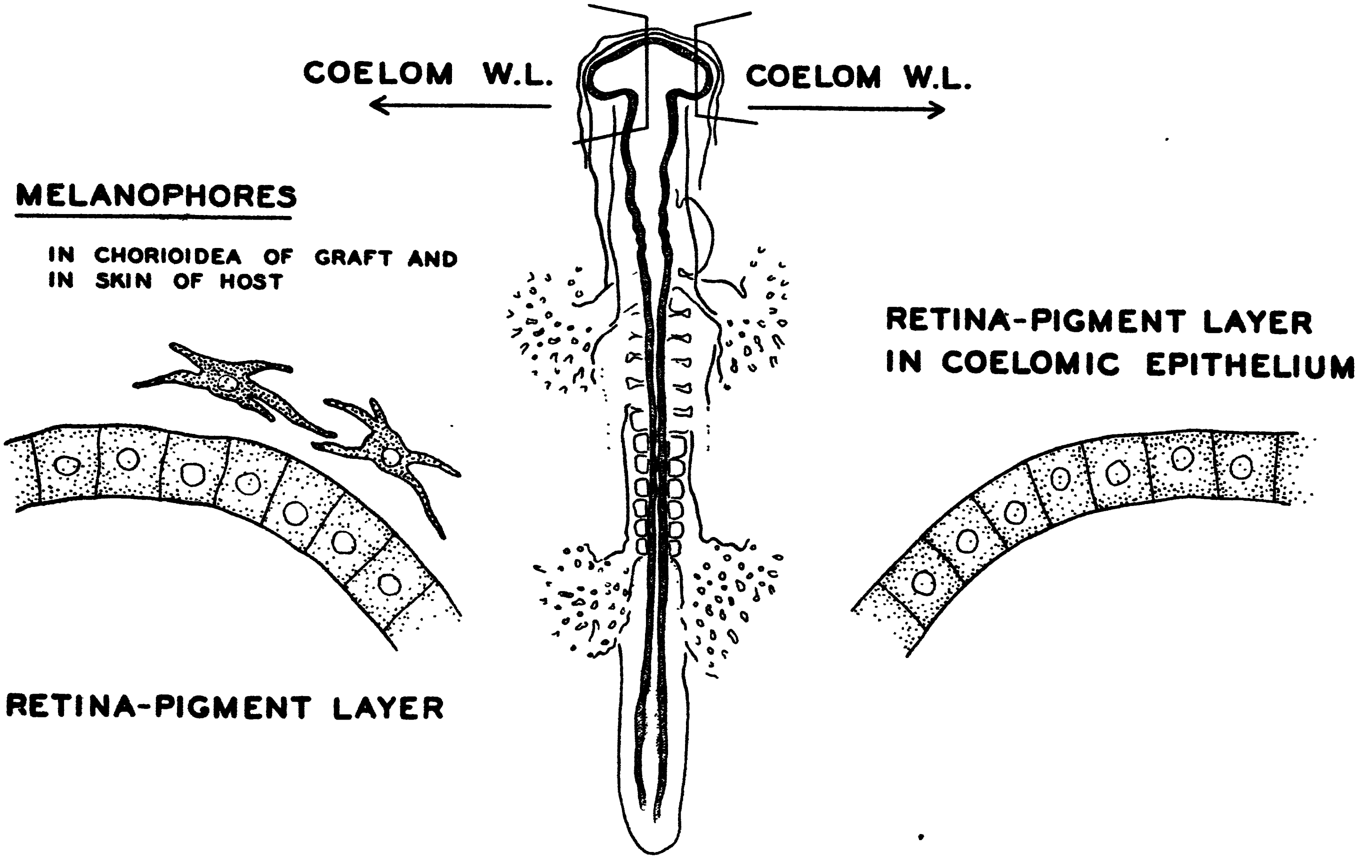

FIG. 17.—Diagram showing relationship of retinal and chorioid pigment to the neural crest (see text and Table 4)

the host. This indicates that retinal epithelium is not capable of producing melanophores. This is confirmed by the result of the next experiment.

Another problem is that of the origin of the two types of pigment cells. The similarity of the chorioid melanophores to those of the skin suggests their origin from the neural crest. What then is the relation of the retinal pigmentation to the neural crest? To answer these questions two types of experiments were carried out (Fig. 17): (1) Eye vesicles of Barred Rock embryos from 6-12 somites were isolated before the neural-crest cells could have migrated in and transplanted into the coelom of 60–70-hour White Leghorn embryos. (2) Eye vesicle with forebrain wall of similar embryos, containing neural crest, were transplanted in the same manner.

The results are given in Table 4. When the optic vesicle does not contain neural-crest cells, it forms a clump of tissue with folded retinal pigment epithelium. No melanophores, however, are found (Table 4, cases 48-2; 48-7; 48-9; 48-10). On the other hand, if the graft contains neural-crest cells, a great number of melanophores is observed in the chorioidea of the grafted eye which developed in the coelom, as well as in the peritoneum, skin, and feathers of the host (see cases 48-4; 48-12; and Fig. 10).

The retinal and chorioid pigments in the chick are therefore shown to be independent in origin as demonstrated already in tissue culture by Bisceglie and in our coelomic grafts. One of them may be suppressed while the other one is normally produced as we find it in the White Leghorn. The chorioid pigment cells, characteristic melanophores, are derivatives of the neural crest. The retinal-pigment epithelium, on the other hand, arises in absence of crest cells.

DISCUSSION

I. ORIGIN OF MELANOPHORES

Until recently all theories dealing with the origin of melanophores have been based merely on histological pictures or on the Dopa reaction. It is clear that these methods can reveal only the fate of the pigment cells after the formation of the pigment or the presence of oxidizing enzymes. Any migration of cells before they can be distinguished by these methods was thus left out of consideration. Only the experimental method of isolation and transplantation can test inherent capacities for autonomous pigment formation in the embryonic tissues. This method led to the discovery of the origin of the melanophores from the neural crest in amphibia by Du Shane and in birds by Dorris and by Eastlick and is basic to this work.

Dorris (1936 and 1938) gave evidence from *in vitro* experiments that the neural crest is the source of melanophores in birds. In 1939 she grafted pieces of "neural crest" of black or white breeds into the leg bud of 72-hour embryos. Although the results of these experiments gave strong evidence for the neural-crest origin of the melanophores, sufficient proof was not given. Indeed, neither the development of the neural crest nor its relationship to the production of melanophores in the grafts was discussed. Controls showed only that the pigment was not produced in epidermal or mesenchymal cells. Furthermore, the possibility was left open that other parts of the neural tube might give origin to melanophores. It is impossible to separate the irregular cell strands of the neural crest from the neural tube by the methods used. The grafted pieces, therefore, were not pure neural crest. Consequently, their ability to form pigment is not proof for the derivation of the melanophores from the neural crest alone. Lastly, the method of grafting in the leg bud might be objected to, since a negative case may be due only to a lack of growth of the transplant.

To give sufficient proof that only the neural crest gives rise to melanophores their appearance or absence in transplants would have to be correlated with the morphological development of the neural crest at different ages and at different anterior-posterior levels. It had to be demonstrated that only tissues containing presumptive neural crest, histologically recognizable neural crest, or cells migrating out of the neural crest can produce melanophores. Tissues grafted into the coelom develop into bodies which can be analyzed histologically. Therefore, it was certain in every case whether or not the graft had developed and produced pigment. In the foregoing pages these conditions have been met, and it was shown that the lateral migration of the melanophore-producing potency coincides with the development of the neural crest and that only tissues containing neural crest can produce pigment.

Next the question of the origin of the buff and red-brown pigments arises. These granules as well as the black ones are produced in branched cells, one cell forming only granules of a specific shape and color (Danforth, 1937; Krätzig, 1937). Grafts from Brown Leghorn, pheasant, and robin embryos containing no neural-crest cells give white feathers, whereas grafts containing neural crest always give melanophores characteristic of the donor. These data show that the melanophores producing buff and red-brown granules are derived also from the neural crest. Whether in species having more than one type of melanophores they are different cells, early differentiated, or whether the surrounding tissues, skin, and feather germs or hormones (Krätzig, 1937) influence the kind of pigment they produce, is not yet known (see summary in Fig. 18).

II. MIGRATION OF NEURAL-CREST CELLS AND MELANOPHORES

It has been known for some time that neural-crest cells have the capacity to migrate throughout the body of the embryo, giving origin to such structures as spinal nerves, sheath cells, etc. That the melanophores also have the ability to wander through tissues is made probable by their amoeboid movement which has been observed directly in tissue culture (Dorris, 1936, 1938) and indirectly by the spreading of donor pigment cells in wing-bud grafts (Willier, Rawles, and Hadorn, 1937; Willier and Rawles, 1938, 1940; Rawles, 1939). This migration takes place chiefly in loose tissue, which may explain the great number of melanophores in the mesenchyme. Furthermore, they appear to follow along the surface of dense structures. Neural-crest cells can be seen on the dorsal surface of the dermatome and along the lateral walls of the neural tube, and the fact that melanophores are distributed abundantly along blood vessels has been pointed out throughout the literature. They are often found gathered around them, particularly along the capillaries. This can be seen nicely beneath the coelomic epithelium of a Barred Rock at hatching. But nerves also are followed. This tendency is strikingly shown in those cases where grafts from pigmented breeds produced melanophores in the coelom of White Leghorn chicks. The melanophores, having left the graft, migrate into the peritoneum, accumulate around blood vessels, nerves, and urinary and sex ducts and follow these structures a long way in the host.

In the chick of a black breed at hatching we find a distinct line in the umbilicus where the melanophores seem to accumulate but never pass. A section of this part shows a sudden change in the arrangement of the mesenchyme cells. In the body wall and mesenteries these cells are nearly parallel to one another. At the border to the umbilicus

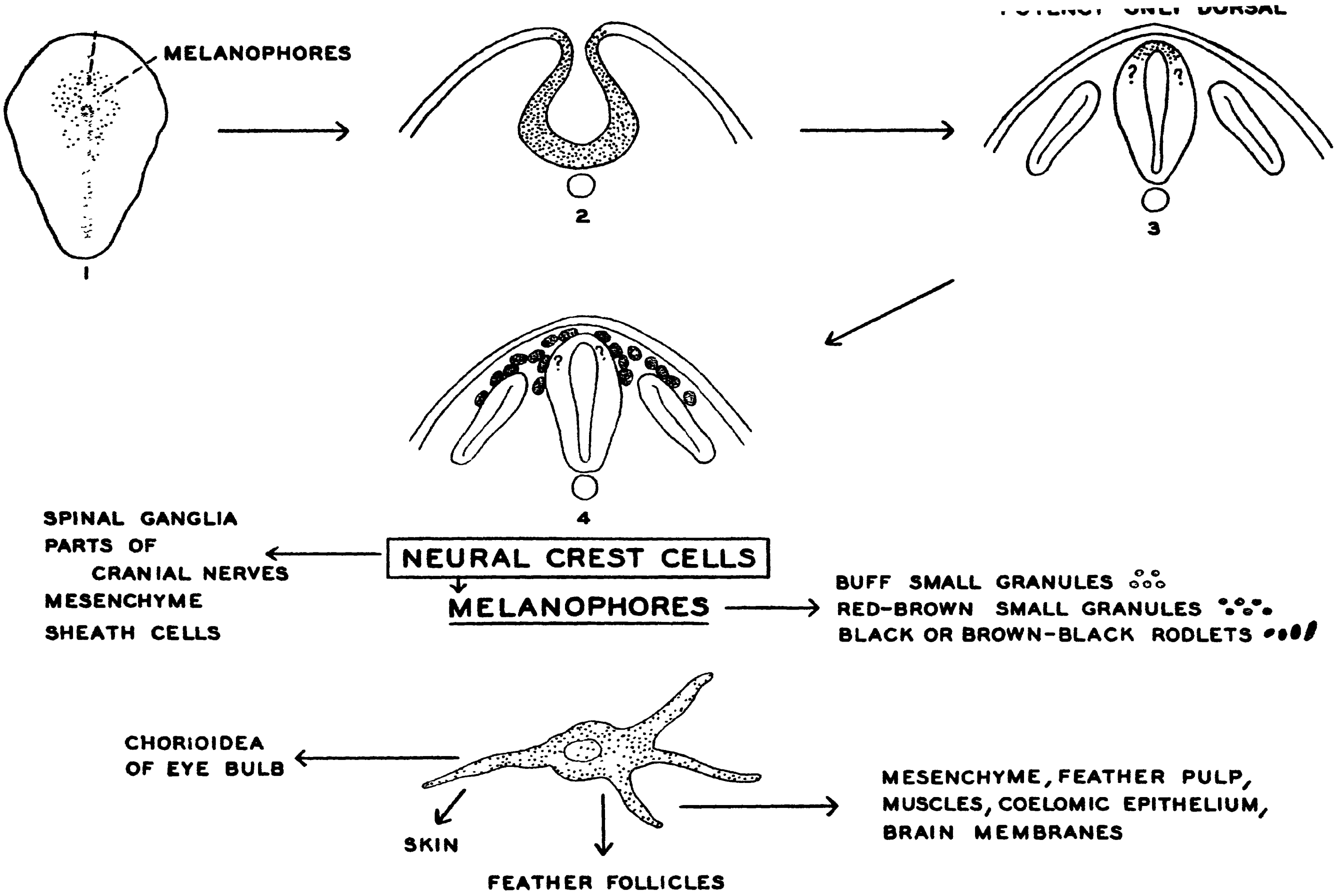

FIG. 18.—Diagram showing the gradual restriction of the melanophore-producing potency. 1. Head-process stage. The melanophore-producing potency coincides with area forming nervous system. 2. During closure the whole neural tube has melanophore-producing potency. 3. The potency to produce melanophores is restricted to the roof. 4. The melanophore-producing potency is localized in the migrating neural-crest cells.

they become very irregular, crossing in all directions and apparently forming a barrier to the migration of the melanophores. In explaining unpigmented regions, we must therefore consider the possibility of mechanical hindrances to the migration of pigment cells.

Eastlick (1938 and 1939*b*) found that grafts in a pigmented embryo were free of host melanophores if attached to the mesentery, from which he concluded that the melanophores did not cross this structure. Similar experiments and an investigation of the pigmentation of chicks at hatching throw more light on this problem and lead to the following results: (1) The mesenteries in Brown Leghorn, Barred Plymouth Rock, and F_1 hybrid in a hatching chick do not contain melanophores. Therefore, an unpigmented graft attached only to the mesentery of the pigmented host remains free of melanophores. (2) Melanophores are able to migrate into the mesentery, if pigmented grafts are attached to them (Fig. 9). Skin and coelomic epithelium, however, remain free of donor pigment in these cases. (3) Donor melanophores migrate into the peritoneum and skin only if a melanophore-producing graft is attached to the body wall. They appear in the mesenteries only if the graft is attached to them. Often a graft is attached to both, leading to a donor pigmentation of skin, peritoneum, and mesentery. From these observations the conclusion is reached that something must inhibit the migration of melanophores into the mesentery during normal development. The same factors hinder the melanophores from leaving the mesentery when provided with a pigmented graft. Comparing this with the situation in the umbilicus suggests a mechanical barrier, i.e., the arrangement of cells prevents the migration of melanophores.

Another problem to consider is the direction of migration of the neural-crest cells. Do they migrate only laterally or also in a longitudinal direction? In Barred Plymouth Rock embryos (50 hours) the neural tube was cauterized over a length of several somites. Three chicks so treated hatched and showed a dorsal opening of about 10 mm., where the spinal cord was destroyed. Yet the skin and mesenchyme all around contain melanophores, demonstrating that the neural-crest cells migrated also in a longitudinal direction. This possibility has to be considered in those experiments where extirpations of the neural crest over a certain length are made to prove the crest origin of any cell type.

It has been suggested that the spread of pigment in embryonic grafts from pigmented to unpigmented breeds and the gradual lateral expansion of the pigment-producing agent in the developing embryo might be due to a diffusion of some substance necessary for pigment production. No evidence supporting this hypothesis has been found. To the contrary, the following facts show that the spreading is due to a migration of pigment-producing cells: (1) The fact that grafts develop on the chorio-allantois or in the coelom of pigmented breeds without producing pigment excludes the presence of such a substance in the blood stream. (2) Except in the retina all melanin is produced in a specific type of branched cell having the ability to migrate through the tissues. (3) These cells are shown to be, in all probability, derivatives of neural-crest cells. The lateral spread of the melanophore-producing agent corresponds to the outgrowth of cells from the neural crest. (4) In grafts from one breed of chick to another and from pheasant, pigeon, and robin to chicks pigment granules peculiar to the donor are formed in melanophores. It is very improbable that a "diffusing substance" could produce donor-specific granules in host cells. (5) An irregular arrangement of mesenchyme cells apparently forms a mechanical barrier for the spread of pigmentation. It is hardly possible that this could be a hindrance for a diffusible substance.

III. PIGMENT FORMATION AND NERVOUS SYSTEM

Rawles and Willier (1939) and, independently, Eastlick (1939*a*) demonstrated that in primitive-streak and head-process stages the area producing nervous system in chorioallantoic grafts coincides with that giving rise to melanophores. In later development Hensen's node always gives pigment in grafts (Watterson, 1938), as also do portions of the most posterior neural tube, as shown in the foregoing pages. As development proceeds, the ventral part of the neural tube loses the melanophore-producing potency. Whether this determination is due to a separation and migration of cells or to an actual change in the cells is difficult to decide.

In all these experiments with different birds and with all types of melanin (buff, red-brown, and black)—contrary to the statements of Klee (1889), Lloyd-Jones (1915), and Greite (1934)—no evidence was found for the production of melanophores in epidermal or mesenchymal cells. Only derivatives of the nervous system can give origin to melanin, viz., the retinal-pigment layer and the melanophores derived from the neural crest.

In mammals the origin of the melanophores (melanoblasts of Bloch) from the neural crest is very probable but has not yet been demonstrated. The more recent investigators assume that epidermal cells form melanin independently of the branched pigment cells. But all their evidence is based on purely histological studies and the Dopa reaction. Whether the epidermal cells of mammals are really able to produce melanin pigment independent of melanophores can be established only by early isolation and transplantation of mammal skin and mesenchyme.

IV. THE HOMOLOGY OF VERTEBRATE MELANOPHORES

Branched melanin-producing cells have been observed in the epidermis and in many mesodermal structures of all the vertebrate groups. Their morphological similarity is striking. They all possess the capacity to migrate, and their invasion into the epidermis from the mesoderm has been described for all classes. In cold- and warm-blooded groups—viz., amphibians and birds—their origin from the neural crest is now demonstrated. Yet one great difference exists between the lower and the higher vertebrates. Fishes, amphibians, and reptiles are able to change their color owing to the movement of the chromatophores and of the pigment granules in the cells themselves. The pigment is carried by living cells which are influenced by nervous and hormonal stimulations, by temperature and by light. The skin of birds and mammals, however, is covered by dead epidermal structures—feathers and hair which serve as temperature insulators. Movements of melanophores in the skin would give no effect on the appearance of the coats of warm-blooded vertebrates. Yet in mammals the melanophores are still approached by nerve fibers as Sasybin (1933) has shown. Whether this holds true for birds has yet to be demonstrated. The difference between the melanophores in lower and in higher vertebrates seems to be due, therefore, largely to the fact that dead cells of hairs and feathers affect the coloration in birds and mammals. Their agreement in form, in melanin-production relationship to the nervous system and hormones, in migration, and, above all, their origin from the neural crest (amphibians and birds) leads to the conclusion that they are homologous cells. In view of this homology the same name should be applied to these cells in all classes. In lower vertebrates they are usually called melanophores—the melanin-producing type of chromatophore. Bloch (1930) called them melanoblasts in mammals to distinguish them from another type of cell—the melanophores which he thought unable to produce melanin but capable of carrying it over from melanoblasts to epidermal cells. Since the existence of such cells is still hypothetical, the older name "melanophore" has been given preference in this work.

If we are allowed to generalize from the conditions obtaining in amphibians and birds we reach the conclusion that in all groups of vertebrates the melanin pigmentation in its great variety is produced by one type of cell—the melanophores. These are derived from the neural crest and migrate into the various pigmented parts of the animal, particularly the skin and its structures.

SUMMARY

1. By isolating and transplanting portions of various pigmented fowl and other bird embryos from different levels along the embryonic axis to the wing bud, coelom, and chorio-allantoic membrane of host chick embryos it is demonstrated that (1) the melanophores originate outside of the epidermis or mesenchyme, migrating into them at a certain stage of development, and (2) only tissues containing presumptive neural crest or neural-crest cells which have migrated into them (as revealed by histological study) give rise to melanophores when transplanted to White Leghorn embryos. Thus, if isolated early enough, i.e., before the neural-crest cells migrate in, grafts from pigmented breeds develop skin and feathers without pigment.

2. By a histological study of the control side of the donor embryo it has been possible to correlate the appearance of melanophores in the graft with the morphological development of the neural crest at the time the isolation was made.

3. By dividing segments of the neural tube into dorsal and ventral halves and transplanting each separately into the wing bud or coelom of White Leghorn host embryos, it is shown that (1) the dorsal halves containing either presumptive or morphologically observable neural crest give rise to melanophores and (2) the corresponding ventral halves do not produce melanophores.

4. Experiments were made to determine the origin of the retinal and chorioid pigments of the eye and their relationship to the neural crest. The pigment of the retina is produced in every cell of the pigmented layer and is not derived from the neural crest. The chorioidea contains pigment in characteristically branched cells, the melanophores, which are identical with those in the skin and mesoderm and, like them, derived from the neural crest.

5. From the experimental evidence now accumulated for various pigmented fowl—pheasant, Black Fantail Pigeon, and robin—it is highly probable that all melanin-producing cells are derived from neural-plate material—the retinal pigment from the forebrain, the melanophores from migrating neural-crest cells.

6. The possible homology of the melanophores in all vertebrate groups, indicated by their agreement in development and by their morphological and physiological characters, is discussed.

LITERATURE CITED

BISCEGLIE, V. 1932. Studi sui tessuti espiantati. IV. Richerche sulle cellule pigmentate della coroide e della retina coltivate in vitro. Zeitschr. f. Zellforsch., 16:228.

BLOCH, B. 1930. Ueber das melanotische Pigment. Rev. suisse de zool., 37:283.

CASPARY, H. 1891. Ueber den Ort der Bildung des Hautpigmentes. Arch. f. Dermat. u. Syph., 23:3.

DANFORTH, C. H. 1937. Pigment cells in heterogenous feathers. Anat. Rec., 68:461.

DORRIS, F. 1936. Differentiation of pigment cells in tissue cultures of chick neural crest. Proc. Soc. Exper. Biol. and Med., 34:448.

———. 1938. The production of pigment *in vitro* by chick neural-crest. Roux' Arch. f. Entw'mech. 138:323.

———. 1939. The production of pigment by chick neural crest in grafts to the 3-day limb bud. Jour. Exper. Zoöl., 80:315.

Du Shane, G. P. 1934. The source of pigment cells in Amphibia. Anat. Rec., 60:62.

———. 1935. An experimental study of the origin of pigment cells in Amphibia. Jour. Exper. Zoöl., 72:1.

Eastlick, H. 1938. A study of pigmentation in the chick embryo by means of limb bud transplantations. Collecting Net, 13:116.

———. 1939*a*. The pigment forming capacity of the blastoderm of Barred Plymouth Rock embryos as shown by transplants to White Leghorn hosts. Anat. Rec., 73, Suppl. 2, 64.

1939*b*. The point of origin of the melanophores in chick embryos as shown by means of limb bud transplants. Jour. Exper. Zoöl., 82:131.

Ehrmann, S. 1885. Untersuchungen über die Physiologie und Pathologie des Hautpigmentes. Arch. f. Dermat. u. Syph., 17:507.

Greite, W. 1934. Die Strukturbildung der Vogelfeder und ihre Pigmentierung durch Melanine. Zeitschr. wiss. Zool., 145:283.

Haecker, V. 1890. Ueber die Farben der Vogelfedern. Arch. f. mikr. Anat., 35:68.

Hamburger, V. 1938. Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. Jour. Exper. Zoöl., 77:379.

Harrison, R. G. 1910. The outgrowth of the nerve fiber as a mode of protoplasmic movement. Jour. Exper. Zoöl., 9:707.

Holmdahl, D. E. 1928. Die Entstehung und weitere Entwicklung der Neuralleiste (Ganglienleiste) bei Vögeln und Säugetieren. Zeitschr. f. mikr.-anat. Forsch , 14:99.

Holtfreter, J. 1933. Der Einfluss von Wirtsalter und verschiedenen Organbezirken auf die Differenzierung von angelagertem Gastrulaektoderm Roux' Arch. f. Entw'mech , 127:619.

Jarisch, A. 1892. Ueber die Bildung des Pigmentes in den Oberhautzellen. Arch. f. Dermat. u. Syph., 24:223.

Jones, D S. 1939. Studies on the origin of sheath cells and sympathetic ganglia in the chick. Anat. Rec., 73:343.

Kerbert, C. 1877. Ueber die Haut der Reptilien und anderer Wirbeltiere. Arch. f mikr. Anat., 13:205.

Klee, R. 1886. Bau und Entwicklung der Federn. Hallersche Zeitschr. f. Naturwiss.. 59:110.

Kolliker, A. 1860. Ueber die Entstehung des Pigmentes in den Oberhautgebilden. Wurzburger naturw. Zeitschr., 1.

———. 1887. Ueber die Entstehung des Pigmentes in den Oberhautgebilden. Zeitschr. f. wiss. Zool., 45:713.

Krätzig, H. 1937. Histologische Untersuchungen zur Frage der Struktur- und Farbveränderungen an Federn nach künstlicher (Thyroxin) Mauser. Roux' Arch. f. Entw'mech., 137:86.

Lewis, W. H. 1903. Wandering pigmented cells arising from the epithelium of the optic cup, with observations on the origin of the M. sphincter pupillae in the chick. Amer. Jour. Anat., 2:405.

Lloyd-Jones, O. 1915. Studies on inheritance in pigeons. II. A microscopical and chemical study of the feather pigments. Jour. Exper. Zoöl., 18:453.

Mertsching, A. 1889. Histologische Studien über Keratohyalin und Pigment. Virchows Arch. f. path. Anat., 116:484.

Post, H. 1894. Ueber normale und pathologische Pigmentierung der Oberhautgebilde. Virchows Arch. f. path. Anat., 135:479.

Rawles, M. E. 1939. The production of robin pigment in White Leghorn feathers by grafts of embryonic robin tissue. Jour. Genetics, 38:517.

Rawles, M. E., and Willier, B. H. 1939. The localization of pigment producing potency in presomite chick blastoderms. Anat. Rec., 73, Suppl. 2, 43.

Riehl, G. 1884. Zur Kenntnis des Pigmentes im menschlichen Haar. Arch. f. Dermat. u. Syph., 11:33.

Rous, P., and Murphy, I. B. 1911. Tumor implantations in developing embryo. Jour. Exper. Med., 24:1.

Sasybin, N. 1933. Ueber die Innervation der Pigmentzellen bei Säugetieren. Zeitschr. f. Zellforsch., 20:476.

Strong, R. M. 1902. The development of color in the definitive feathers. Bull. Mus. Comp. Zoöl., 40: 147.

STRONG, R. M. 1915. Further observations on the origin of melanin pigments. Anat. Rec., **9**:128.

SZILY, A. V. 1911. Ueber die Entstehung des melanotischen Pigmentes im Auge der Wirbeltierembryonen und in Choroidealsarkomen. Arch. f. mikr. Anat., **77**:87.

WATTERSON, R. 1938. On the production of feather color pattern by mesodermal grafts between Barred Plymouth Rock and White Leghorn chick embryos. Anat. Rec., **72**, Suppl. 100.

WEIDENREICH, F. 1912. Die Lokalisation des Pigmentes und ihre Bedeutung in Ontogenie und Phylogenie der Wirbeltiere. Zeitschr. f. Morph. u. Anthrop., Sonderheft, **2**:59.

WILLIER, B. H. 1924. The endocrine glands and the development of the chick. Amer. Jour. Anat., **33**:67.

WILLIER, B. H., and RAWLES, M. E. 1938. Feather characterization as studied in host-graft combinations between chick embryos of different breeds. Proc. Nat. Acad. Sci., **24**:446.

———. 1940. The control of feather color pattern by melanophores grafted from one embryo to another of a different breed of fowl. Physiol. Zoöl. **13**:177.

WILLIER, B. H.; RAWLES, M. E.; and HADORN, E. 1937. Skin transplants between embryos of different breeds of fowl. Proc. Nat. Acad. Sci., **23**:542.

WINCKLER, F. 1910. Studien über Pigmentbildung I. und II. Roux' Arch. f. Entw'mech., **29**:616.

PLATE I

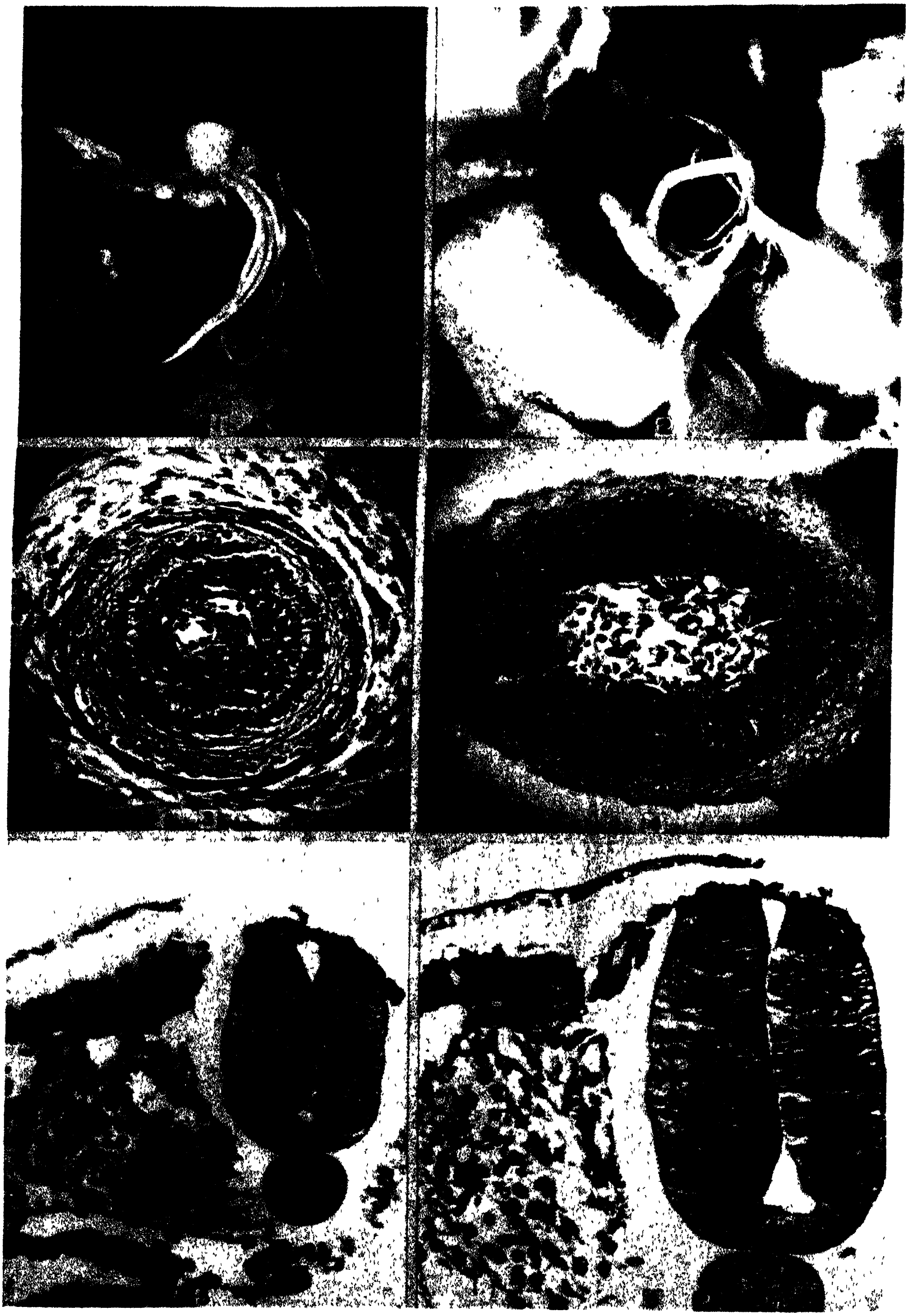

PLATE I

FIG. 1.—Coelomic graft with white down feathers, obtained from a piece of lateral plate of a 16-somite Ringnecked Pheasant, cut between neural tube and somites and implanted into the coelom of a White Leghorn. No melanophores developed (case 55-6).

FIG. 2.—Vesicle of skin containing black and white down, grown in the coelom of a White Leghorn host, derived from head skin of a Barred Plymouth Rock embryo of 37 somites. Note on the left side the donor melanophores in the body wall of the White Leghorn host (case 7-2).

FIG. 3.—Cross-section through a feather germ of the transplant shown in Fig. 1. No melanophores present. Bouin, haematoxylin-eosin.

FIG. 4.—Cross-section through a feather germ developed from a piece of lateral plate at the level of the twenty-fifth somite of a Barred Rock embryo of 31 somites, grafted to the chorio-allantois of a Barred Rock embryo. Note the presence of melanophores (case 34-8).

FIG. 5.—Cross-section of left side of donor Barred Plymouth Rock embryo of 23 somites at the level of the tenth somite. The corresponding right side was cut at the edge of the neural tube, thus excluding neural-crest cells, and grafted to the chorio-allantois of a White Leghorn. No melanophores developed in the graft (case 39-2.) The cutting caused the slight distortion seen on the right side of the tube.

FIG. 6.—Cross-section of left side of donor Barred Plymouth Rock embryo of 31 somites at the level of the twenty-fifth somite. The corresponding right side was cut between the neural tube and the somites, thus including migrating neural-crest cells, and grafted to the chorio-allantois of a Barred Plymouth Rock. The graft developed melanophores (case 34-8).

PLATE II

FIG. 7.—Intracoelomic graft (*G*) developed from the ventral half of the neural tube of a Barred Rock of 18 somites at the level of the fourteenth somite. Note the absence of melanophores (case 47-3).

FIG. 8.—A piece of neural tube from a Barred Rock of 13 somites developed in the coelom of a White Leghorn host. The implant was cut at the level of the sixth somite. Note the melanophores in the graft (*G*) and the body wall of the host (case 20-5).

FIG. 9.—Intracoelomic graft (*G*) from ventral half of the neural tube of an F_1 hybrid of 30 somites isolated at the eleventh somite level. The ventral half at this level contained neural-crest cells. Note the melanophores which migrated into the mesentery. No melanophores were found in the body wall or skin of the White Leghorn host (case 40-8).

PLATE II

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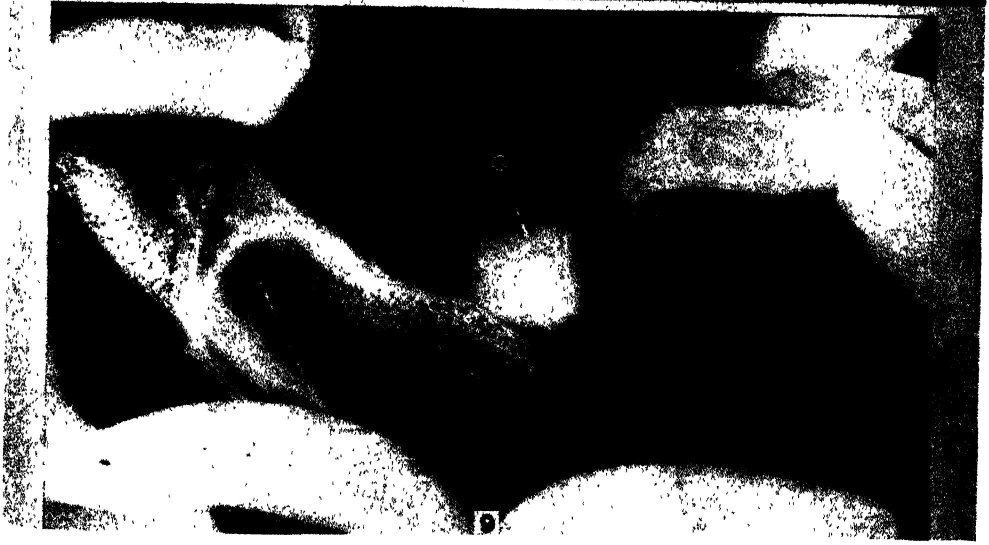

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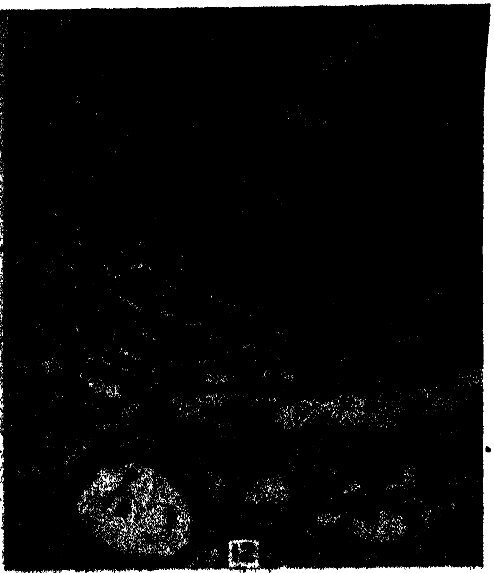
PLATE III



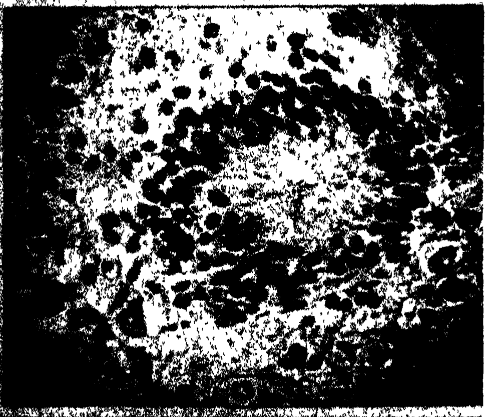


PLATE III

FIG. 10.—Abnormal eye vesicle (*E*) with pigmented layer of retina and pigmented chorioid coat grown in the coelom of a White Leghorn. The implant contained one side of the telencephalon plus the corresponding optic vesicle of a Barred Rock of 11 somites. The chorioidea contains melanophores, some of which have also migrated into the body wall of the host (case 48-12).

FIG. 11.—Epithelium of retina with black pigment developed from a piece of an eye bulb of a Barred Rock of 8 somites in the coelom of a White Leghorn embryo. No melanophores present (case 48-7).

FIG. 12.—Section of a graft from the dorsal half of the neural tube, cut at the level of the eighth somite of a Barred Plymouth Rock of 35 somites and implanted into the coelom of a White Leghorn. Note neural tube (?) and melanophores in mesenchyme below (case 42-2).

FIG. 13.—Section of a coelomic implant developed from the ventral half of the neural tube of a Mongolian Pheasant of 26 somites cut at the level of the twenty-fourth somite. Note mass of ganglion cells, nerve fibers, and the absence of melanophores (case 56-5).

FIG. 14.—Section of a coelomic graft obtained from a piece of an eye vesicle of a 6-somite Barred Rock. Note retinal pigment and absence of melanophores (case 48-2).

THE KARYOPLASMIC RATIO IN THE EMBRYOS OF MONOVOLTINE AND BIVOLTINE RACES OF THE SILKWORM BOMBYX MORI L.

(Four figures)

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RACES of *Bombyx mori* L. are known which have one generation a year (monovoltine), two (bivoltine), or several *generations* (polyvoltine). The monovoltine moths lay eggs in summer that first show a period of embryonic development of 2–3 days prior to the formation of the embryonal stripe, the serosa, and the amnion. The monovoltine eggs remain in this stage during late summer, autumn, and winter; the following spring they resume their development and hatch. Under normal conditions the period of morphological quiescence continues 9 months or more. While experimentally this may be shortened or prolonged, it is always necessary for the eggs to pass through a period of low temperature—but not longer than 2 or 3 months—at 6°–7° C.

The development of polyvoltine eggs is continuous; they hatch within 10–20 days after oviposition, according to the temperature. The polyvoltine races are present in tropical countries.

The cells of the serosa of the monovoltine eggs are charged with pigment, while those of the polyvoltine are not pigmented. The bivoltine races have the first generation in the spring, laying eggs that develop immediately. The serosa of these is without pigment, as in the case of polyvoltine eggs. The moths produced from such eggs lay eggs that develop as do those of the monovoltine races, having pigmented serosa, a period of morphological quiescence during autumn and winter, and requiring a period of low temperature. All monovoltine races are more or less variable; some eggs develop as do bivoltine races, while the reverse may be observed in the bivoltine laying.

The phenomenon of monovoltinism and bivoltinism has been the subject of extensive investigations. Some authors believed that the phenomenon was strictly due to the climate, so that the monovoltine races transferred from temperate to tropical climates became bivoltine, and vice versa; but monovoltinism, bivoltinism, and polyvoltinism do not now appear so easy to explain, and workers are not yet in agreement regarding the meaning of all the observed phenomena. It is known with certainty only that some environmental conditions do facilitate bivoltinism and polyvoltinism and some do not; that each race may shown an internal tendency to various forms of voltinism;[1] and that the environmental conditions may produce different effects according to the vital stage of the insect, i.e., high temperatures during incubation are probably opposed to bivoltinism and favorable to monovoltinism (Mollo, 1921, mentioned by Foa, 1927). Actually, we may say that the tendency to bivoltinism or monovoltinism is also dependent upon internal conditions that act in harmony with the environmental conditions determining the degree of voltinism.

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[1] From a bivoltine race Foa (1932) was able to select a monovoltine race.

In this work a continuance of the author's investigations on the physiology of *B. mori* L., a fact that may be connected with the biological mechanism of voltinism, is made known.

Foa (1927), studying the results of Kiyoshi Katsuki (1918), observed that this author found the nuclei of the germinal cells in a bivoltine race of greater volume than those of a monovoltine race; Foa thought in consequence that the karyoplasmic ratio must be different according to the voltinism. She examined the serosa of various races but because of technical difficulties was unable to observe differences in the volume of the nuclei. Scatizzi (1936) confirmed the difference in volume between the monovoltine and the bivoltine germinal cells.

In "Chinese white," "Italian yellow" (monovoltine), and "Awoiko" (bivoltine) Foa (1927) observed that the embryonal stripe (germ band) of bivoltine eggs has larger nuclei (in a simple microscopical observation), the nuclei of the monovoltine Chinese white being smaller and those of the Italian yellow the smallest, though she did not measure the exact size of the cells and of the nuclei. The present author wishes to explain more fully the problem of measuring the nucleoplasmatic quotient of the embryonal stripe of monovoltine and bivoltine races.

MATERIAL AND TECHNIQUE

The author employed monovoltine eggs of Italian yellow race from Padua and bivoltine eggs of Awoiko race from moths bred in Portici (Naples). The eggs were fixed with Carnoy, the Carnoy and the chromoacetic mixture being probably the best fixing liquid for the eggs with the chorion.

The shells were removed from the fixed eggs with Rusma (Niceta, 1929). They were then imbedded in paraffin, cut, and the sections (5 μ) stained with haematoxylin Carazzi and eosin. For measurement the author selected the best sections. The eggs examined were in diapause, before wintering.

Landi (1934) measured the karyoplasmic ratio of silkworm eggs during incubation and the total nitrogen; the karyoplasmic ratio has been expressed by Landi by the formula $NP \times 100/NT - NP$, in which NP is the nitrogen of the aminopurinic bases and NT the total nitrogen. This quotient increases during incubation from 2.30 to 2.96–3.06 at the close of the incubation period.

The karyoplasmic ratio measured by Landi is not comparable to that obtained by measuring the nuclear and cellular volumes, because Landi analyzed the entire contents of the eggs, including the yolk.

RESULTS[2]

The cells of embryonal stripes (germ bands) have nuclei with a visible membrane, chromatin divided in granulation, and cytoplasm lightly granular.

The measurements were made either directly with a micrometric ocular or indirectly from photomicrographs of some good sections at the same magnification.

The volume of the nuclei has been calculated as follows. The ectodermal and mesodermal nuclei are very clearly delimited and appear as elipsoids; the two diameters of these were measured, and the nuclear volume was then calculated.

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[2] The earlier results of my investigations on the karyoplasmic ratio were privately announced to Dr. Foa, who kindly published my letter in her work of 1937.

In practice the author calculated the volume of an elipsoid for each stripe, the diameters of which were the average of the diameters of all the nuclei observed (i.e., the greater diameter was the average of the greater diameters of all the nuclei; the smaller diameter was the average of the smaller diameters). The volume of the ectodermal and mersodermal nuclei was calculated separately. Finally, the author obtained the average of the average nuclear volumes of each stripe.

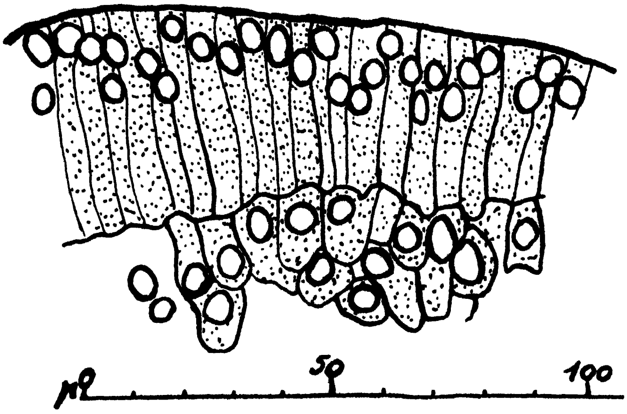

Fig. 1.—Semischematic drawing of a region of the embryonal stripe of an Italian yellow egg in diapause. From a photomicrograph.

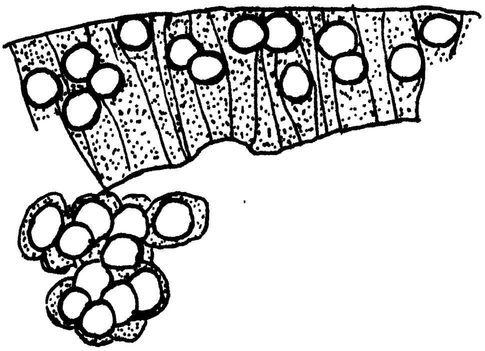

Fig. 2.—Semischematic drawing of a region of the embryonal stripe of an "Awoiko" egg (bivoltine) in diapause. From a photomicrograph. (The drawing and photomicrographs are all made with the same magnification. Cf. the scale of Fig. 1.)

The volume of the ectodermal cells was indirectly calculated as if cylindrical, taking as their diameters the smaller diameter of the ectodermal nuclei and as their length the height of the stripe. It may be clearly observed in Figures 1–4 that the cellular boundaries are often tangent to the nuclear boundaries. The cells are really not regular cylinders, but in the eggs observed the ectoderm is formed by a monostratified epithelium. The length of the cells occupies the entire height or thickness of the stripe, and the nuclei generally appear in a line toward the amniotic cavity. Some ectodermal nuclei appear

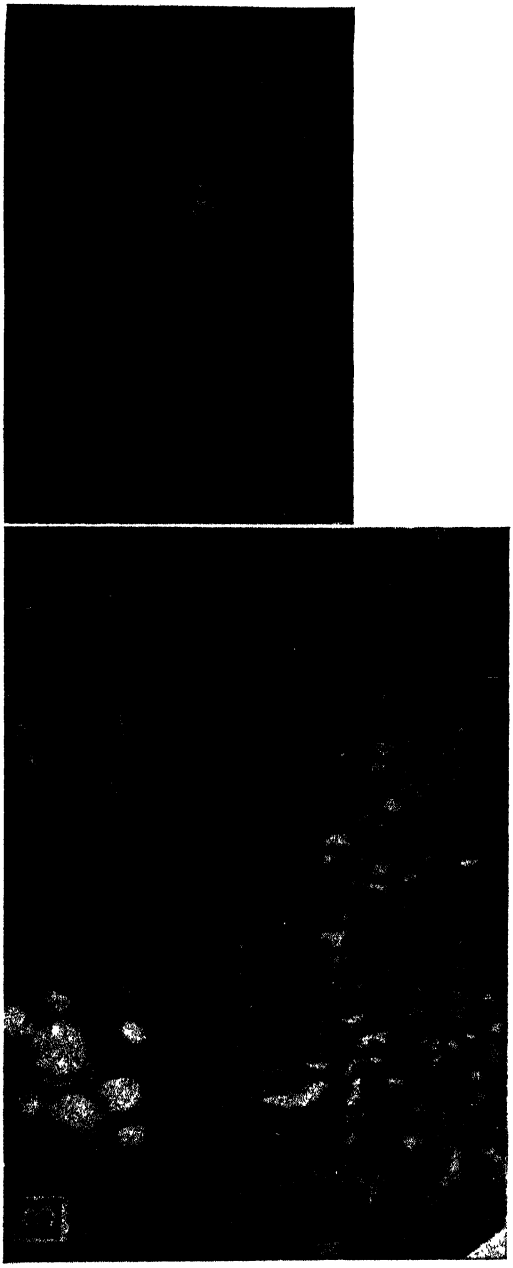

FIG. 3.—Photomicrograph of cells of the embryonal stripe of a monovoltine race (Italian yellow)

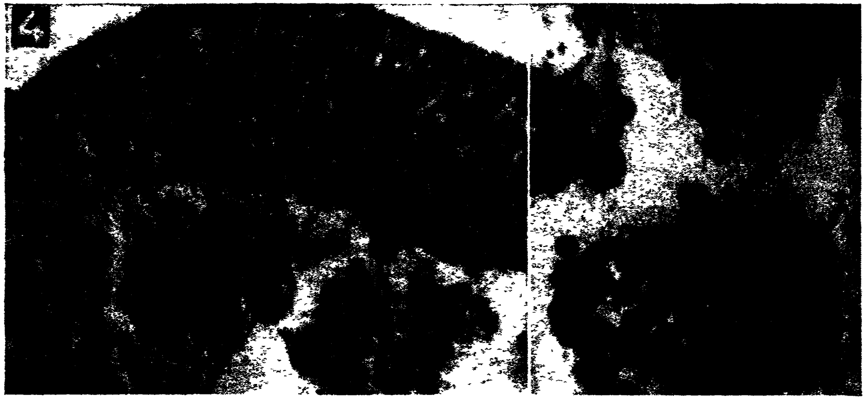

FIG. 4.—Photomicrograph of cells of the embryonal stripe of a bivoltine race (Awoiko). Observe the smaller volume of the nuclei compared with those of Figure 3.

toward the mesoderm; but it may also be observed that the corresponding cells extend for the total height of the stripe, from the mesoderm to the amniotic boundary.

The nuclear volume has been compared to the volume of the entire cell or to the volume of the cell diminished by the nuclear volume. The mesodermal cells are somewhat irregular in form although roughly spherical, and their volume was calculated by considering the cells as spheres. Two or more diameters of each cell were measured, and the volume was calculated with the average of the various diameters.

TABLE 1

THE NUCLEOPLASMATIC RELATIONSHIP

A. SIZE AND VOLUME OF THE NUCLEI

Nuclei	Smaller Diameter	Greater Diameter	Volume
Monovoltine:			
Ectodermal	4 78 μ	5 96 μ	71 24 μ^3
Mesodermal . . .	4 83	5 62	68.74
Bivoltine:			
Ectodermal .	6 79	8 42	205 50
Mesodermal .	6 80	8 79	212 75

B SIZE AND VOLUME OF THE CELLS

Cells	Average Height	Average Width	Volume of Total Cell	Volume of Cytoplasm
Ectodermal:				
Monovoltine .	36 89 μ	4 78 μ	1029 1 μ^3	958 0 μ^3
Bivoltine	35 8	6 79	1298 49	1093 0
Mesodermal:				
Monovoltine	13 07	13 07	1168 61	1099 87
Bivoltine.	12 00	12 00	904 61	691 85

C. RELATIVE VOLUME OF THE NUCLEI
(Karyoplasmic Ratio)

	Cell Volume	Nuclear Volume	Volume of Cytoplasm	Nuclear Volume
Monovoltine:				
Ectoderm	100	6 92	100	7.43
Mesoderm. .	100	5 88	100	6 24
Bivoltine:				
Ectoderm.	100	15 82	100	18 8
Mesoderm.	100	23 51	100	30 75

The diameter of the mesodermal cells is about 2.5 times greater than the average diameter of the mesodermal nuclei in the Italian yellow; in the bivoltine eggs the diameter of the mesodermal cells is about 1.65 times that of the average diameter of the mesodermal nuclei. The general results are summarized in Tables 1 and 2.

The mesodermal bivoltine cells have very little cytoplasm. In some stripes the nuclei of these cells appear as if surrounded by only a fine stratum of cytoplasm. Further measurements with a more exact method, such as comparing with a planimeter the cellular and nuclear surfaces, should give absolute results slightly different from those indicated; but the relative results will not be changed materially.

TABLE 2

CELL MEASUREMENTS

Number	Nuclei (Average Diameter)		Embryonal Stripe
	Ectoderm	Mesoderm	Average Height of Cells
	Embryonal Stripe, Monovoltine (Italian Yellow "Ascoli")		
1	5.58	5.35	39.62
2	5.20	4.51	38.55
3	5.96	5.58	42.53
4	5.27	4.36	30.90
5	5.27	4.89	33.35
6	4.74	...	41.31
7	5.50	5.04	37.79
8	5.04	...	40.69
9	5.27	...	...
10	5.27	5.81	35.03
11	5.35	5.20	37.63
12	5.04	...	32.13
13	5.73	5.20	35.34
14	5.81	4.89	31.05
15	5.27	5.43	30.75
16	5.35	5.66	33.50
17	4.81	4.05	40.23
18	5.66	6.04	40.69
19	6,04	5.96	38.25
20	5.12	5.73	38.55
21	5.35	5.12	39.93
	Embryonal Stripe, Bivoltine (Awoiko)		
11	8.01	8.38	...
12	7.19	7.43	31.93
23	8.12	7.68	...
24	6.42	7.12	38.43
26	7.75	8.39	37.22

Table 2 also indicates that the form of the nuclei is variable; the mesodermal nuclei are more rounded than the ectodermal in monovoltine eggs. The ectodermal nuclei, moreover, may be more or less elongated according to the eggs. In the bivoltine eggs examined the ectodermal and mesodermal nuclei appear more rounded than the corresponding nuclei of the monovoltine eggs; in bivoltine eggs the author has not observed such a considerable difference in form between the mesodermal and the ectodermal nuclei of the monovoltine stripe.

In the bivoltine eggs we have also observed one or two very large nucleoli; in monovoltine eggs the nucleoli are smaller or absent. A nuclear and cellular variableness may also occur in the same stripe. Not only is the karyoplasmic ratio greater in the bivoltine races, but so is the absolute volume of the ectodermal and mesodermal cells, in comparison with the monovoltine races. We may suppose that the higher metabolism of the bivoltine races is correlated with the relatively greater volume of the nuclei.

SUMMARY

In the embryonal stripe (germ band) of a monovoltine race of *B. mori* L. (Italian yellow Ascoli) the karyoplasmic ratio is greater during the diapause than in a bivoltine race (Awoiko) in both the mesodermal and the ectodermal cells. There is also variability in form and volume of the mesodermal and ectodermal nuclei in the eggs of each race considered.

LITERATURE CITED

FOA, A. 1927. Osservazioni ed esperienze sul bivoltinismo del baco da seta. Ann. R. Ist. agrario Portici (3d ser.), 2:3.

———. 1932. Una razza annuale di bachi da seta ottenuta da una bivoltina soltanto con la selezione. *Ibid.*, Vol. 5.

———. 1937. Accrescimento e sviluppo nelle razze bivoltine del *Bombyx mori* L.; probabile correlazione tra l'andamento di questi fenomeni e il rapporto nucleo-protoplasmatico delle cellule germinali. Boll. zool, Anno 8, Nos. 1–2.

KATSUKI, KIYOSHI. 1918. Cytologische Studien über die Samenzellen von Seidenraupen. Bull. Im. Sericicultural exper. sta. Japan, Tokyo, Vol. 1, No. 2.

LANDI, E. 1934. Le Variazioni dell'azoto purinico e il cosidetto rapporto nucleo-plasmatico nelle uova del *Bombyx mori* L. Ann. R. staz. bac. sper., Padova, 47:23.

MOLLO, A. 1921. Influenza della temperatura di incubazione del seme sulla conservazione del bivoltinismo. Informaz. ser., Milano, Vol. 8, No. 17.

NICETA, F. 1929. Contributo alla tecnica per lo studio delle uova degli insetti. Atti. Soc. ital. Sc. nat., Vol. 68.

SCATIZZI, I. 1936. Primi dati sulla differenza di dimensioni delle cellule germinative in razze bivoltine e univoltine di *Bombyx mori* L. Boll. Soc. ital. biol. sper., Vol. 11.

TIRELLI, M. 1931*a*. Contributo allo studio dell'azoto ammidico nelle uova di *Bombyx mori* L. Arch. di farmacol. sper. e sc. aff., 43(5):115.

———. 1931*b*. Aumento di viscosità del succo delle uova di *B. mori* in seguito a riscaldamento. Zeitschr. f. vergl. Physiol., 14:737.

1931*c*. Il Comportamento del glicogeno nelle uova del *B. mori* durante lo sviluppo embrionale. *Ibid.*, 15:148.

——. 1931*d*. Una ipotesi spaziale e meccanica della blastocinesi degli insetti. Zool. Jahrb., Abt. Physiol., 49:59.

——. 1931*e*. Fisiologia degli insetti. Fenomeni chimici e chimico-fisici nell'uovo del *B. mori* L. Viscosità, stratificazione, struttura e funzione delle sfere vitelline. Ann. R. staz. bac. sper., Padova, 46:308.

——. 1934. Studi su alcuni fenomeni fisiologici nelle uova ibride del *B. mori* L. Zool. Jahrb., Abt. Physiol., 53:367.

1936. Per la migliore conoscenza degli stadi embrionali tipici illustrati dal grandori nel *B. mori.* Arch. zool. ital., 21:485.

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THE ROLE OF THE INJURED SURFACE IN THE PRODUCTION OF THE INJURY POTENTIAL OF FROG MUSCLE

(Two figures)

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WHEN one end of a striated muscle is injured, an electrical potential difference can be measured between injured and uninjured regions. This potential difference has been termed the injury potential. It is customary to regard this injury potential as due to the potential difference across the uninjured surface of the muscle fiber, the injured end merely serving as an indifferent lead to the inner protoplasm. Some evidence has been adduced to show that the injured surface plays a more important role in the production of the injury potential in invertebrate muscles (Steinbach, 1933); and Sugi (1934) has published figures showing that the magnitude of the injury potential of frog muscle can be altered by changing the concentration of the medium in contact with the injured surface.

Recent experimental work and theoretical considerations (Steinbach, 1935; Teorell, 1935; Meyer and Sievers, 1936) emphasize the role of all electrolyte phases in contact with membranes and particularly the protoplasmic electrolytes (Steinbach, 1935*a*). From our knowledge of the chemical constituents of muscle protoplasm it is possible to predict that diffusion potentials should arise between protoplasm and the usual biological environments and that these potential differences should account for a considerable portion of the measured injury potential, regardless of the nature of the membranes involved.

If diffusion tendencies of the protoplasm play an important role in the production of the injury potential, then diffusion potentials should arise at both injured and uninjured surfaces. In other words, the magnitude of the injury potential should be dependent upon the concentration of electrolytes in the solutions applied at either injured or uninjured surfaces. Sugi (1934) presents potential difference/log. concentration curves to show that this is, in general, true for frog muscle; but complete results are not given, and it is difficult to evaluate his findings. Since diffusion potentials have been shown to be of importance at both injured and uninjured surfaces in scallop muscles (Steinbach, 1933), it seemed essential to study further their role in frog muscle. Experiments relating to this are presented here.

METHODS

Sartorius muscles of common frogs (mostly *Rana pipiens*) were excised with as little injury as possible. In most cases they were immersed for about half an hour in Ringers fluid and then for another half-hour or hour in isotonic buffered sucrose solution. This latter step was for the purpose of washing out excess extracellular electrolytes—a procedure found to be essential for consistent results in the electrical measurements. The sucrose solution was buffered with sodium phosphate mixtures, about 0.0005 M, at pH approximately 7.5. All salt solutions were similarly buffered.

In measuring injury potentials a fresh cut was made at the proximal end of the muscle, and the tissue then placed on a paraffin block so formed that the cut end could just make contact with fluid in one depression of the block, the uninjured surface being immersed in fluid in another depression (Fig. 1). A vaseline seal was placed between injured and uninjured surfaces of contact in such a fashion that only the cut surface was touched by the solution. Electrical contact to the solutions was made by saturated KCl bridges in contact with calomel half-cells. Care was taken to avoid contamination of the solutions by the bridges. Potential-difference measurements were made with a vacuum-tube voltmeter of high-input resistance.

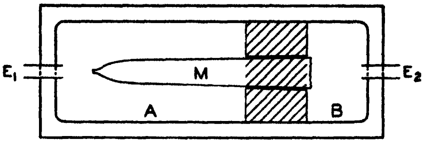

FIG. 1.—Diagram of arrangement of muscle on the paraffin block. The muscle (*M*) is situated so the cut end dips into solution *B*, while the uninjured end is immersed in solution *A*. The vaseline seal is shown by cross-hatching. E_1 and E_2 are salt bridges leading to the nonpolarizable electrodes and voltmeter.

RESULTS

The system measured may be represented by the following chain with reference to Figure 1: electrode and bridge^{a}/ solution A^{b}/ uninjured muscle^{c}/ injured muscle^{d}/ solution B$^{a'}$/ electrode and bridge. The potential differences at *a* and *a'* are equal and opposite, hence the total measured potential difference will be due to other regions of the chain. The two interfaces that can be experimented with most easily are those represented as *b* and *d*—the uninjured and the injured surfaces. Asymmetry potentials might arise between injured and uninjured protoplasm as represented or perhaps due to the asymmetry of the membrane (*b*) itself (cf. Francis, 1937).

If the total injury potential is due, in the main, to diffusion tendencies of the protoplasmic electrolytes, then alteration of the salt concentration present in either fluid *A* or fluid *B* should have a considerable effect on the measured potential difference. Changes in potential difference as the salt concentration is altered will be termed "concentration potentials." Sugi (1934) has offered curves showing such concentration potentials, and his results find a ready explanation in terms of diffusion potentials. In my own work I have found it impossible to reproduce exactly the shape of the curves shown by Sugi, largely because of the fact that changes in potential difference due to concentration changes at the uninjured surface are only slowly reversible. Hence, the results reported here will not be offered as precise log. concentration/p.d. curves but will be concerned, in the main, with a comparison of the concentration potentials as salt concentrations at *A* and *B* are changed from 0.124 to 0.002 N or the reverse. Two salt solutions were used, KCl and NaCl. Dilutions were made with isotonic sucrose as described. Typical experiments are described in Figure 2. Representative values taken from such experiments are given in Table 1.

It is apparent that the magnitude of the injury potential is controlled by the concentration of KCl at the injured surface as well as at the uninjured. NaCl at the injured surface gives nearly the same concentration potential that KCl does, but a much lower one at the uninjured surface.

These results are invariable, provided that sufficient time is given for the equilibrium values to be established (as much as half an hour or more for NaCl at the uninjured surface) and provided that the excess extracellular electrolytes are washed out before starting the experiment. That this last procedure should be important is not surprising, since rather high diffusion potentials arise between NaCl solutions of different concentrations, and this factor added to the "short-circuiting effect" would greatly complicate the interpretation of the results.

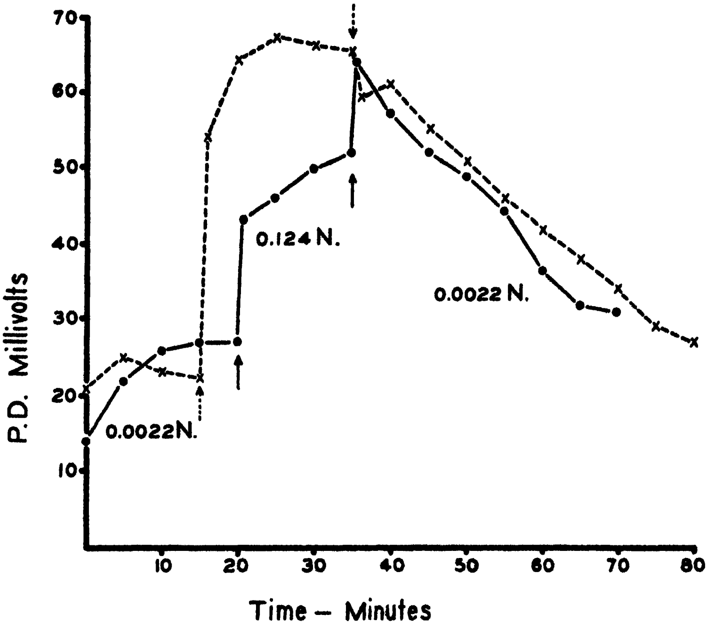

FIG. 2.—Typical experiments on two muscles showing concentration potentials (change in potential difference as the external concentration is changed) at the cut surface. In both cases the initial solutions were 0.0022 N in salt at both ends. Solutions changed at cut surface at the times indicated by arrows. - - - X - - -, KCl as salt. —— • ——, NaCl as salt.

TABLE 1*

CONCENTRATION POTENTIALS IN FROG SARTORII AS MEASURED BETWEEN THE SOLUTIONS INDICATED

Frog Muscle		Solution	Concentration Potential (Millivolts)	
			Cut Surface	Uninjured Surface
1	1	NaCl	+57	+18
	2	KCl	+54	+61
2	1	NaCl	+51	+22
	2	KCl	+43	+56

* Concentrations used: 0.124 N and 0.0022 N. Dilute solution applied first in all cases. Plus signs refer to dilute solution.

The effects of salt concentration on injured and uninjured surfaces result, of course, in different injury potentials, depending on the composition of the leading-off fluids. This is shown by the figures in Table 2, which represent injury-potential measurements observed during the course of the same experiment given in Table 1. With isotonic NaCl at both ends of the muscle, a high injury potential is obtained. With dilute NaCl at both ends, the injury potential is much lower. With isotonic KCl at both ends, the injury potential is very low, and with dilute KCl is somewhat higher.

TABLE 2

INJURY POTENTIALS OF THE MUSCLES SHOWN IN TABLE 1 WITH EITHER CONCENTRATED OR DILUTE SOLUTIONS AT BOTH ENDS OF MUSCLE AS SHOWN

Frog	Muscle and Salt	Injury Potential (Millivolts)	
		0.002 N Both Ends	0.124 N Both Ends
1	1 NaCl	+9*	+48
	2 KCl	+14	+7
2	1 NaCl	+10	+39
	2 KCl	+21	+8

* Plus signs refer to uninjured surface as measured in the external circuit.

DISCUSSION

Several characteristics of the injury potential of frog muscle have been known for a long time. Among these may be mentioned the fact that potassium is particularly potent in reducing the value of the measured injury potential when applied to the uninjured surface. Less attention has been paid to the fact that the magnitude of the injury potential is controlled to a certain extent by the salt concentration of the external medium, regardless of the nature of the salt present. This latter point has been noted, however, several times and should be recognized. The results reported here add two other facts that must be taken into consideration in attempting to formulate the mechanisms giving rise to the injury potential (1) Changes in KCl concentration have essentially the same effects at either the injured or the uninjured surfaces. The end result of this is that there is little asymmetry shown between any two points on a muscle bathed in isotonic KCl. (2) Changes in NaCl concentration at the cut surface have essentially the same effect as changes in KCl concentration. Changes in NaCl at the uninjured surface give much lower concentration potentials. This, of course, has as an end result a rather high asymmetry potential with isotonic NaCl at both ends. An injury potential can be measured with Ringers fluid on both cut and uninjured surfaces.

The fact that KCl concentration has the same influence at either surface of the frog muscle suggests strongly that the surfaces become electrically identical under the influence of the high potassium, that is, an uncut surface treated with strong KCl becomes, in effect, an injured surface. This has been recognized experimentally for some time. It is quite customary to produce an injury potential by treating one point on a cell or tissue with isotonic KCl.

On the other hand, the differences in NaCl concentration potentials show clearly that some definite asymmetry must exist in the system. Considering only a very simplified system in which unmodified diffusion potentials arise and in which concentration differences can be maintained by some mechanism, an easy explanation of all these results can be provided. Consider, for example, the following chain which in essence is like that described in Figure 1:

$$\underset{1.0}{\mathrm{NaCl}}/^{b}\ \underset{0.1}{\mathrm{K}X^{c}}/\ \underset{1.0}{\mathrm{K}X^{d}}/\ \underset{1.0}{\mathrm{NaCl}}$$

In this chain, concentrations are noted in relative units, and the NaCl solutions shown represent Ringers fluids at uninjured ($KX = 0.1$) and injured ($KX = 1.0$) surfaces. The membrane thus has only one-tenth the concentration of protoplasmic electrolytes present at the injured surface. If the mobility of the X anion is assumed to be 10, the mobilities of the other ions being as in water, the potential differences at the various interfaces can be calculated and from them the total measured potential difference of the chain. The chain shown above would have an "injury potential" of 51 millivolts (mv.) with the injured ($KX = 1.0$) surface negative. Dilute NaCl (0.02 relative concentration) at the two ends of the chain would give a total potential difference of only about 6 mv. Dilute KCl at the two ends would give a slightly higher result than with dilute NaCl. A concentrated KCl solution at either end would still give a total potential of about 40 mv., but if the membrane concentration is assumed to increase under the influence of the KCl the asymmetry vanishes, and the potential difference between the two ends vanishes. In general, then, such a simplified scheme accounts very well for the observed results if the assumptions are made that the membrane normally has a low electrolyte concentration and that electrolyte concentration increases upon injury (removal of the membrane) or upon KCl treatment.

It should be pointed out that this assumed increase in electrolyte content of the membrane is more strictly an increase in conductivity and could be due either to an actual increase in number of ions present or to an increase in mobility of ions in the membrane, provided the mobility ratios of the ions remained about the same. It seems unlikely that membranes specifically permeable to potassium alone would be operative, since such a membrane is obviously not present at the injured surface, and yet the KCl concentration potentials are the same at both surfaces. The possibility of a selective membrane is not ruled out by these observations, but it is rendered rather unnecessary if it is present. Another factor militating against the presence of a membrane selectively permeable to potassium is the fact that NaCl does have a definite concentration effect at the uninjured surface. It is unlikely that a surface could exist that allowed apparent penetration of NaCl and yet was highly selective for potassium.

The system that can account for the injury potential in terms of diffusion potentials between cellular and extracellular phases involves, as a necessary consequence, a cell surface that can maintain a low electrolyte concentration. Injury removes this layer, and apparently it is not regenerated to any great extent, at least during the times observed in these experiments. Possibly plug formation (see Heilbrunn, 1940) has something to do with this behavior.

Other interfaces than the ones considered in this simplified system are probably present and operative in giving rise to the injury potential. The fact that the injury potential is usually not quite zero when isotonic KCl is bathing both surfaces of the muscle is some indication of this. It might very well be true, for example, that injured protoplasm differs from normal protoplasm and that both are different in electrolyte makeup from the cell surface. If that is so, then there is at least one more interface present in the measured system which could be a source of asymmetry and can contribute to the injury potential.

SUMMARY

It is shown that concentration potentials are developed at both the injured and the uninjured surfaces of frog muscle with NaCl and KCl solutions. The concentration potentials with KCl at both surfaces and with NaCl at the injured surface are all of about the same magnitude and sign. NaCl concentration potentials at the uninjured surface are of the same sign but much lower.

These findings are discussed in terms of diffusion potentials, and it is shown that a simplified scheme postulating no selective membranes is adequate to explain the results.

LITERATURE CITED

FRANCIS, W. L. 1937. The surface membrane of muscle fibers Proc. Roy Soc London, B, 122:140.

HEILBRUNN, L. V. 1940 The action of calcium on muscle protoplasm Physiol. Zoöl., 13:88.

MEYER, K. H., and SIEVERS, J. F. 1936. La Perméabilité des membranes. Helvetia chim. acta, 19:649.

STEINBACH, H B 1933. Injury potentials in scallop muscles. Jour. Cell. and Comp. Physiol., 3:203.

——. 1935*a* Diffusion potentials in scallop muscles. *Ibid.*, 7:771.

——. 1935*b*. Diffusion potentials in colloidal systems. *Ibid.*, p. 291.

SUGI, Y. 1934. On the electromotive effect of salt on muscle. Jap J Med Sci. (Trans. and Abst.), Biophysics III, 3:27.

TEORELL, T. 1935. Studies on the "diffusion effect" upon ionic distribution. Proc. Natl Acad. Sci., 21:152.

THE STIMULATING ACTION OF CITRATES AND OXALATES ON THE NEREIS EGG[1]

(Two figures and two plates)

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TREATMENT of the unfertilized egg of *Nereis limbata* with any one of several physical and chemical agents stimulates the egg to undergo a series of reactions, one of which is the breakdown of the germinal vesicle (Just, 1933; Heilbrunn and Wilbur, 1937; Wilbur, 1939). The process of germinal-vesicle breakdown has proved a convenient reaction for the study of stimulation and response in a response system consisting of a single cell. This response has been thought to be dependent upon the calcium of the egg cortex, for if the egg is first treated for a few minutes with an isotonic solution of sodium citrate, which presumably removes or binds at least a portion of the cortical calcium, the response to the stimulating agents is reversibly inhibited.

This inhibitory action of citrate is not peculiar to marine eggs. The use of citrates and oxalates in preventing the clotting of blood is well known. The following processes are also inhibited by citrates and oxalates or both: muscular contraction (Locke, 1894; Howell, 1894); heart beat (Koch, 1881; Howell and Eaton, 1893; Januschke, 1909; Gros, 1913; Salant and Hecht, 1915; McCartney and Ransom, 1917); intestinal contractions (Salant, Mitchell, and Schwartze, 1916; Salant and Schwartze, 1917); tone and amplitude of contraction of uterus (Sugimoto, 1913; Messini, 1929); tone of artery (Ellinger, 1926); rhythmic movements of stomach (Russo, 1935); vagus action on the heart (Shafer, 1936; Shafer and Crismon, 1936); and ciliary action (Gray, 1922; Wenner, 1931). On the other hand, citrates and oxalates do not always show an inhibitory action but may act as stimulating agents. This has been described for muscle (Locke, 1894; Garrey, 1905; Guenther, 1905; Rebello and Fontès, 1925); nerve (Loeb, 1901; Mathews, 1904; Moore, 1921; Fessard, 1936; Brink and Bronk, 1937; Brink, Sjostrand, and Bronk, 1939); cerebellum (Robertson and Burnett, 1912); heart beat (Russo, 1935); tone and amplitude of contraction of uterus (Sugimoto, 1913; Messini, 1929); contraction of muscles of blood vessels (Russo, 1935); intestinal contractions (MacCallum, 1903, 1904; Salant, Mitchell, and Schwartze, 1916; Salant and Schwartze, 1917; Salant, Kleitman, and Wright, 1922); and marine eggs (Loeb, 1902; Just, 1922). Clotting-time is decreased after injections of sodium citrate (Weil, 1915; Neuhof and Hirshfeld, 1922; Higgins and Fisher, 1924; De Souza and Hocking, 1934, 1935) and oxalic acid (Steinberg and Brown, 1939). Foster (1940) has not been able to confirm the results of Steinberg and Brown.

Now the question arises: If calcium is necessary for stimulation, as so many have thought, why should salts which bind calcium—such as citrates and oxalates—act as stimulating agents? The present experiments were carried out in an attempt to gain some insight into this stimulating action of citrates and oxalates and to decide, if possible, whether or not calcium is also necessary for the stimulating action of citrates and oxalates or whether this type of stimulation is different from the other agents and can occur in the absence of calcium. If, in the *Nereis* egg, the stimulating action of citrates and oxalates should prove to be present after treatment of the egg with 0.35 M sodium citrate—a procedure which normally prevents stimulation—then calcium could no longer be considered a requisite for all types of stimulation. Information gained from a study of the *Nereis* egg as to the mechanism of the stimulating action of citrates and oxalates would presumably contribute to an understanding of the mode of action of these substances in the more complex systems mentioned above.

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[1] This paper constitutes a thesis presented to the faculty of the graduate school of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

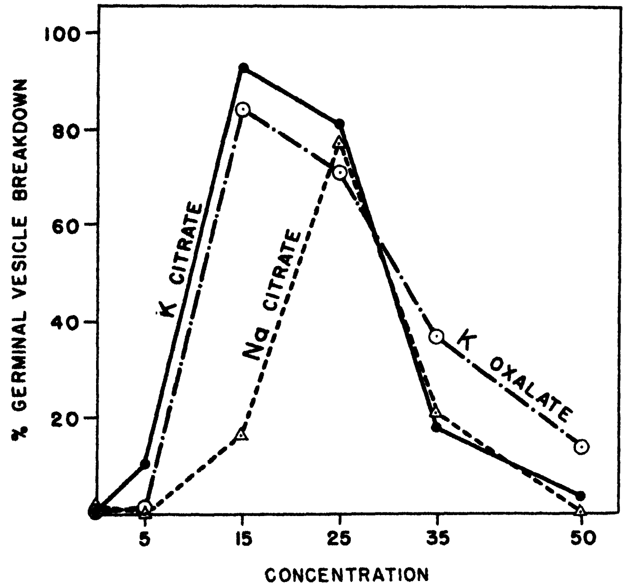

FIG. 12.—Each point represents the average value of 9–12 experiments. The concentration is expressed as parts of citrate or oxalate solution in 100 parts of a mixture of the citrate or oxalate solution and sea water.

METHODS

Eggs from a single female were washed once in sea water and concentrated in the center of a finger bowl containing approximately 250 cc. of sea water. One to 3 drops of eggs were then placed in each of a series of stender dishes containing from 30- to 40-cc. samples of the various concentrations of the substance under consideration. After a period of from 2 to 4 hours eggs were removed from each dish, compressed between a slide and cover slip, and the percentage of germinal-vesicle breakdown determined. At least 100 eggs were counted in each case, with one exception noted later. The points on the curves in Figures 12 and 13 represent the average values of 9 to 12 experiments.

The variation in percentage of germinal-vesicle breakdown between eggs from different females was considerable and is illustrated in the tables of data. In order that the samples counted might be representative, eggs were picked up from different parts of each dish. It is interesting to note that in certain solutions, at least, the eggs showing germinal-vesicle breakdown were not distributed at random. For example, if one observes eggs in dishes containing mixtures of sodium citrate solution and sea water in which a considerable percentage, but not all, of the eggs have undergone germinal-vesicle breakdown, it is frequently seen that the eggs showing germinal-vesicle breakdown are localized in one or more areas. That is, in one region all or nearly all the eggs have undergone breakdown, while in another region nearly every egg may show an intact germinal vesicle. Dr. Josephine Hollingsworth[2] has observed this phenomenon in eggs activated by isotonic salt solutions.

In experiments in which eggs were transferred from one solution to another the amount of solution carried over never exceeded 3 drops. The room temperature at which the experiments were carried out varied between 21.6° and 26.3° C.

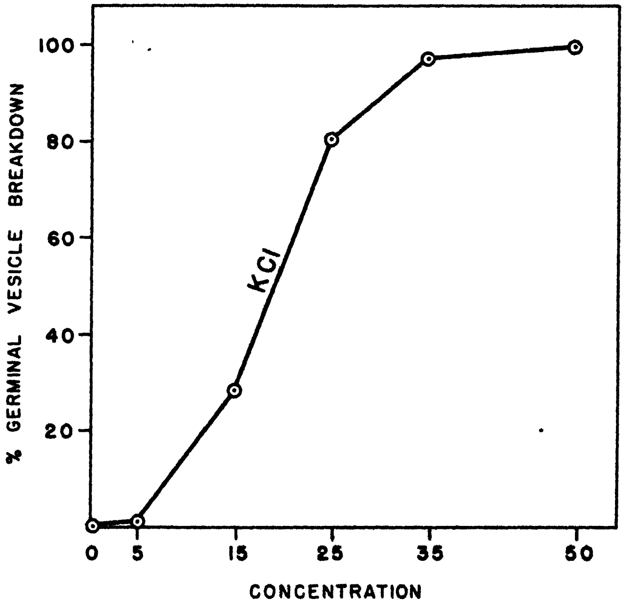

FIG. 13.—Each point represents the average value of 9 experiments

The following concentrations of solutions were employed: KCl, 0.53 M; NaCl, 0.53 M; potassium, sodium, and ammonium citrates, 0.35 M; and potassium and ammonium oxalates, 0.3 M. (Dr. F. Moser has found that a 0.35-M solution of sodium citrate causes neither shrinkage nor swelling of the *Arbacia* egg; and the concentration of citrates used in this study is based on this determination. The value of 0.3 M for the oxalate solutions may not be isotonic with the *Nereis* egg.) These solutions were mixed with sea water in various proportions, and the mixtures will be indicated as percentages of these solutions in sea water. For example, 25 parts of 0.53 M KCl added to 75 parts of sea water is termed a 25 per cent KCl–sea-water mixture. When citrates or oxalates are added to sea water, the osmotic pressure of the mixture will be somewhat different from that of the components taken individually, owing to the partial binding of the calcium and magnesium of the sea water.

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[2] Personal communication.

Throughout the experiments attention was directed primarily to the phenomenon of germinal-vesicle breakdown. However, many interesting cortical and cytoplasmic changes resulting from treatment of the eggs with mixtures of citrates or oxalates with sea water were noted and will be briefly indicated from time to time but will not be discussed in detail.

POTASSIUM SALTS

Figure 12 indicates the percentage of germinal-vesicle breakdown in mixtures of sea water with potassium citrate and with potassium oxalate. The curves for potassium citrate–sea-water mixtures and potassium oxalate–sea-water mixtures are strikingly similar, both showing a high percentage of breakdown in the 15 per cent and the 25 per cent mixtures with much lower values for higher and lower concentrations. In a narrow range, then, mixtures of sea water with potassium citrate and with potassium oxalate have a pronounced stimulating action.

Some batches of eggs immersed in the potassium citrate–sea-water mixtures showed polar-body formation (Fig. 1). This was especially true of the 25 per cent mixture. In the 15 per cent and 25 per cent mixtures the oil drops moved to one side of the egg—a phenomenon which precedes cleavage after normal fertilization. Coalescence of oil drops was indicated by a decrease in number and an increase in the size of the drops. Membranes were present. Figure 2 shows an untreated *Nereis* egg. A small percentage of eggs were cytolyzed in certain mixtures. This was true not only of the potassium citrate mixtures but of the other citrates and oxalates as well.

In the potassium oxalate–sea-water mixtures polar-body formation was most common in the 15 per cent and 25 per cent mixtures but occurred occasionally in the 35 per cent and 50 per cent mixtures. Membranes lifted a considerable distance from the cell periphery were characteristic of the latter mixtures. This type of membrane is much different from that which follows fertilization. Often these membranes were double in character. (Fig. 3). (Cf. Costello and Young, 1939.) Abnormal cleavage is shown in Figures 4, 5, and 6.

Eggs were immersed in mixtures of isotonic KCl and sea water in order to determine whether the potassium ion, quite apart from citrate or oxalate action, was capable of initiating germinal-vesicle breakdown in concentrations of the same order of magnitude as used in the potassium oxalate– and potassium citrate–sea-water mixtures.[3] From the results summarized in Figure 13 it is evident that KCl is potent in producing germinal-vesicle breakdown. Polar-body formation and concentration of oil drops in one region of the egg took place (Fig. 7). In view of these results with KCl–sea-water mixtures the germinal-vesicle breakdown occurring in the potassium citrate– and potassium oxalate–sea-water mixtures cannot be definitely attributed to the effects of the citrate and oxalate ions but may have been due, in part at least, to the action of the potassium ion. However, the KCl mixtures differ from the citrate and oxalate mixtures in that a decrease in the percentage of breakdown does not occur with an increase in the concentration of KCl. A pure isotonic solution for KCl will also give a high percentage of breakdown (Heilbrunn and Wilbur, 1937).

SODIUM SALTS

Average percentages of germinal-vesicle breakdown in sodium citrate–sea-water mixtures are not greatly different from percentages obtained for the potassium citrate– and potassium oxalate–sea-water mixtures with the exception of a lower percentage for the 15 per cent sodium citrate mixture (Fig. 12).

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[3] Although the percentage mixtures were the same for KCl as for potassium citrate and potassium oxalate, the concentration of the potassium ion is different in each case.

Polar-body formation (Figs. 8 and 9), lobulation or budding-off of small portions (Fig. 10), and cleavage (Figs. 9 and 11) were characteristic of the 25 per cent mixture. Lobulation was also present in the 35 per cent mixture; and in certain batches some of the eggs cleaved in to several blastomeres (?) which were roughly similar in size. Coalescence of oil drops took place (Figs. 8, 9, and 11). A pigmented area was often prominent (Figs. 8, 9, and 11; see also Fig. 6).

Eggs immersed in NaCl–sea-water mixtures of the same concentration as those employed with KCl mixtures showed, with very few exceptions, little or no germinal-vesicle breakdown. This was true of eggs left in these mixtures 12 hours or longer. Nine batches of eggs were studied. The results indicate that the stimulating action of sodium citrate cannot be brought about solely by the sodium ion but that the stimulation must be related to the citrate ion.

AMMONIUM SALTS

Ammonium citrate and ammonium oxalate solutions mixed with sea water were much less effective than the other citrates and oxalates studied in producing germinal-vesicle breakdown. The concentrations used were the same as those employed for the previously mentioned citrates and oxalates. The highest percentage of germinal-vesicle breakdown was found in the 25 per cent ammonium oxalate mixtures, which showed an average of 25.3 per cent for 12 batches of eggs. Three batches showed high percentages of breakdown in certain concentrations. These particular mixtures had been prepared for 7 and 8 days before use. Freshly prepared solutions did not show this stimulating action. Both polar-body formation and cleavage were almost completely absent in the ammonium citrate and oxalate mixtures. However, the eggs from a single female did show abnormal cleavage in 25 per cent, 35 per cent, and 50 per cent ammonium oxalate mixtures. Single and double membranes were characteristic of the 50 per cent ammonium oxalate–sea-water mixtures.

Germinal-vesicle breakdown was almost completely absent in ammonium citrate mixtures. A "browning" of the eggs occurred in all concentrations of the ammonium citrate mixtures. In a 50 per cent mixture many or all of the eggs showed this at the end of 2 hours of immersion. Eggs in a 5 per cent mixture did not exhibit this brown coloration to any marked degree after 2 hours, but here, too, many or all became brown a few hours later.

INHIBITORY ACTION OF CITRATES

Previous work had demonstrated that a few minutes' treatment of the *Nereis* egg with 0.35 M sodium citrate would reversibly inhibit the stimulating action of isotonic NaCl, KCl, and ultra-violet irradiation (Heilbrunn and Wilbur, 1937). This suggested an interesting experiment. The data cited above show that citrate–sea-water mixtures act like other stimulating agents in producing germinal-vesicle breakdown. If this stimulating action of the citrate–sea-water mixtures is comparable to the stimulating action of the NaCl, KCl, and ultra-violet irradiation, then a pretreatment with 0.35 M sodium citrate should also inhibit the stimulating action of the citrate–sea-water mixtures. The experiment has been carried out, and it has been found that the 0.35 M sodium citrate does indeed reversibly inhibit the stimulating action of the sodium citrate–sea-water mixtures. Table 1 gives the results of such experiments in which eggs were immersed in

0.35 M sodium citrate for 6 or 10 minutes and then transferred to 15 per cent and 25 per cent sodium citrate–sea-water mixtures. The inhibition of breakdown in 25 per cent mixtures following treatment with 0.35 M sodium citrate is especially marked (last two columns of table). Experiments 2 and 11 provide exceptions to the inhibition in the 15

TABLE 1

INHIBITORY ACTION OF 0.35 M SODIUM CITRATE

No. of Expt.	Immersion Time in 0.35 M Sodium Citrate (Minutes)	Percentage Breakdown in a 15 Per Cent Sodium Citrate Sea-Water Mixture	Percentage Breakdown in a 15 Per Cent Sodium Citrate-Sea-Water Mixture Following Citrate Treatment	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture	Percentage Breakdown in a 25 Per Cent Sodium Citrate-Sea-Water Mixture Following Citrate Treatment
1	6	97	21	98	0
2	6	2	52	92	16
3	6	98	16	99	0
4	6	94	10	96	8
5	6	11	0	99	21
6	10	52	40	98	10
7	10	95	40	98	12
8	10	29	19	91	0
9	10	5	16	60	6
10	10	73	1	84	8
11	10	59	78	94	10
12	10	7	11	66	18
13	10	80	67	97	18
14	10	86	42	98	1
15	10	52	51	88	6

TABLE 2

INHIBITORY ACTION OF 0.35 M POTASSIUM CITRATE

No. of Expt.	Immersion Time in 0.35 M Potassium Citrate (Minutes)	Percentage Breakdown in a 15 Per Cent Potassium Citrate-Sea-Water Mixture	Percentage Breakdown in a 15 Per Cent Potassium Citrate-Sea-Water Mixture Following Citrate Treatment	Percentage Breakdown in a 25 Per Cent Potassium Citrate-Sea-Water Mixture	Percentage Breakdown in a 25 Per Cent Potassium Citrate-Sea-Water Mixture Following Citrate Treatment
1	6	. . . .	0	. .	0
2	6		0	. .	0
3	6	. . .	0	. . .	0
4	6	100	0	1	0
5	6	100	0	86	2
6	6	100	0	97	0
7	6	94	1	84	0
8	6	99	0	57	0
9	6	100	0	35	0

per cent mixture. Here it is seen that germinal-vesicle breakdown is greater in eggs treated with 0.35 M sodium citrate before immersion in the 15 per cent mixture than in untreated eggs placed in the same solution. These will be discussed later. Experiments 9 and 12 appear to be similar, although the increased breakdown following treatment with 0.35 M citrate may be within the experimental error of counting. This behavior was never encountered in the 25 per cent mixture of sodium citrate or in either the 15 per cent or the 25 per cent mixtures of potassium citrate (Table 2). Six minutes' immersion in 0.35 M potassium citrate inhibited the stimulating action of 15 per cent and 25 per cent mixtures of potassium oxalate.

TABLE 3

REVERSIBILITY OF THE INHIBITORY ACTION OF 0.35 M SODIUM CITRATE

No. of Expt.	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture Following 10 Min. Treatment with 0.35 M Sodium Citrate	Percentage Breakdown in a 25 Per Cent Sodium Citrate–Sea-Water Mixture Following Immersion in 0.35 M Sodium Citrate, 25 Per Cent Sodium Citrate–Sea-Water Mixture, and Sea Water	Remarks
1	94	64	76	Eggs immersed in 25 per cent sodium citrate–sea-water mixture for 1 hour, 15 minutes; then transferred to 30–40 cc. of sea water for 15 minutes
2	100	9	91	Same
3	100	21	60	Same
4	99	32	45	Same
5	96	8	8	Eggs immersed in 25 per cent sodium citrate–sea-water mixture for 20 minutes. Other conditions unchanged
6	98	8	46	Same
7	95	2	37	Same
8	90	0	87	Eggs immersed in 25 per cent sodium citrate–sea-water mixture for 20 minutes; then transferred to 200–250 cc. of sea water for 30 minutes
9	33	2	31	Same
10	90	33	89	Same
11	75	5	68	Same
12	98	59	81	Same
13	87	41	85	Same

The eggs which have been inhibited by the 0.35-M citrate are still capable of responding to stimulation after the citrate has been removed, indicating that the 0.35-M solution did not inhibit the reaction by injuring or killing the eggs. The eggs were first treated as previously described; that is, they were placed in 0.35 M sodium citrate followed by the

25 per cent sodium citrate–sea-water mixture. They were then put into sea water to remove the citrate and again placed in the 25 per cent mixture. Table 3 shows that the inhibitory action of the 0.35 M is reversible, for the eggs respond to the final 25 per cent sodium citrate mixture after immersion in sea water. The conditions under which the last 5 experiments were carried out gave almost complete reversibility (see last column of Table 3).

Similar experiments showed the inhibitory action of potassium citrate to be reversible. A 15 per cent mixture was used. Since only 3 drops of eggs were used in the first solution, the loss of eggs in successive transfers did not always permit the counting of 100 eggs in the final solution of potassium citrate and sea water.

Germinal-vesicle breakdown in 15 per cent potassium oxalate is also inhibited if the eggs are first immersed in 0.3 M potassium oxalate for 3–6 minutes. However, the 0.3 M potassium oxalate is unsatisfactory in certain respects. For example, some of the eggs become very dark after a few minutes in this solution, making the determination of the presence of a germinal vesicle difficult and uncertain. An attempt was made to ascertain whether or not the inhibitory effect of 0.3 M potassium oxalate was reversible. The procedure was similar to that described for sodium and potassium citrate. In 5 series carried out there was no indication that the inhibitory action of potassium oxalate is reversible. In 2 series all the eggs were either very dark or completely ruptured in the final solution.

DISCUSSION

The results have shown that the *Nereis* egg, like several other systems mentioned above, is stimulated by citrates and oxalates. In appropriate concentrations in sea water certain citrates and oxalates cause the *Nereis* egg to undergo germinal-vesicle breakdown and to give off polar bodies. Isotonic NaCl and KCl solutions and ultra-violet irradiation also exert a stimulating action on this cell. To suppose that the same series of reactions is initiated by all these stimulating agents would seem more reasonable than to postulate a different mechanism for each agent. And, indeed, these agents are similar in that the stimulating action of each is reversibly inhibited if the eggs are first treated briefly with 0.35 M citrate.[4] In view of this inhibiting action of a calcium-binding compound, it is tempting to believe that calcium is concerned whenever the egg is stimulated. This point of view has been stressed by Pasteels (1935, 1938).

The suggestion has been made (Heilbrunn and Wilbur, 1937; Wilbur, 1939) that a stimulating agent acting upon the egg upsets the normal equilibrium of the egg in one way or another and calcium is released from a calcium-proteinate compound in the egg cortex. The free calcium may then initiate the series of changes which lead to germinal-vesicle breakdown and other maturation processes. The same hypothesis may also be applied to the stimulating action of the mixtures of sea water and citrate or oxalate. Without attempting to specify the changes which these solutions might produce, one may suppose that the normal equilibrium of the egg is altered by these mixtures and that calcium is available to the extent that the processes of germinal-vesicle breakdown, polar-body formation, etc., can take place. The inhibitory action of the stronger concentrations of citrate on such a scheme would be due to a more rapid or more complete removal of calcium with a consequent absence of stimulation. Guenther (1904) believed that the stimulating action of sodium oxalate on muscle was in some way dependent upon the presence of the calcium ion. Loeb (1901) proposed that citrate by removing calcium from the outer layers of a muscle fiber increased the irritability. As calcium diffused outward and replaced that removed by citrate, the fiber became less irritable. Heilbrunn (1940) thinks that citrates and oxalates may remove magnesium from the cell faster than calcium and that the upset in the normal calcium-magnesium ratio which results produces stimulation.

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[4] The reversibility of the action of 0.35 M potassium citrate in inhibiting a potassium oxalate–sea-water mixture has not been tested.

It will be remembered that treatment of eggs with 0.35 M sodium citrate does not always inhibit germinal-vesicle breakdown on transfer to a 15 per cent sodium citrate–sea-water mixture. On the other hand, 0.35 M potassium citrate does prevent the action of a 15 per cent potassium citrate–sea-water mixture. The explanation of this difference seems to be indicated in the experiments with mixtures of sea water with NaCl and KCl in which it was found that the sodium ion is much less potent than the potassium ion in initiating germinal-vesicle breakdown. In terms of the hypothesis suggested above, this would mean that the sodium ion is less effective than the potassium ion in releasing bound calcium. If the sodium ion is not able to release as much calcium, then, perhaps, sodium citrate will remove or bind less calcium than potassium citrate; or, in other words, after treatment with sodium citrate an egg will contain more available calcium than after treatment with potassium citrate. And if sufficient calcium is available, then treatment with a citrate–sea-water mixture will result in stimulation.

From the present discussion it is evident that at present no complete explanation of the stimulating action of citrates and oxalates can be given. The point of view presented here has been that citrates and oxalates, like other stimulating agents, in some manner bring about an increase in free calcium within the cell. The fact that the stimulating action of citrate and oxalate is inhibited when the cell is first immersed for a few minutes in a pure solution of citrate indicates that a sufficient concentration of calcium must be present in the cell even for a response to these calcium-binding compounds, and, accordingly, the stimulating action of citrates and oxalates is not inconsistent with the view that calcium is always necessary for the response of the *Nereis* egg to stimulation.

SUMMARY

1. Solutions of sodium citrate, potassium citrate, or potassium oxalate mixed with sea water in certain proportions stimulate the *Nereis* egg to undergo germinal-vesicle breakdown, polar-body formation, and other changes.
2. The response of the egg to stimulating concentrations of mixtures of sodium or potassium citrate and sea water can be reversibly inhibited by first treating the eggs for a few minutes with 0.35 M sodium or potassium citrate. The inhibitory action of 0.3 M potassium oxalate was not found to be reversible.
3. Solutions of ammonium citrate or ammonium oxalate mixed with sea water have little stimulating action. Ammonium citrate–sea-water mixtures produce a "browning" of the eggs.
4. Mixtures of isotonic KCl and sea water cause germinal-vesicle breakdown and polar-body formation.
5. Mixtures of isotonic NaCl and sea water are much less effective than KCl–sea-water mixtures.

This work was carried out under the direction of Doctor L. V. Heilbrunn. I am indebted to him for his kind suggestions concerning the preparation of the manuscript, to Doctor C. A. Angerer for helpful discussion of the problem, and to Mr. L. Santamaria for translation of an Italian reference. I gratefully acknowledge a grant from the University of Pennsylvania chapter of Sigma Xi.

LITERATURE CITED

Brink, F., and Bronk, D. W. 1937. Proc. Soc. Exper. Biol. and Med., **37**:94.
Brink, F.; Sjostrand, J.; and Bronk, D. W. 1939. Amer. Jour. Physiol., **126**:442.
Costello, D. P., and Young, R A. 1939. Collecting Net, **14**:209.
DeSouza, D., and Hocking, F. D. M. 1934. Jour. Physiol., **83**:49.
———. 1935. *Ibid.*, **85**:168.
Ellinger, F. P. 1926. Pflüger's Arch. f. d. ges. Physiol., **211**:548.
Fessard, A. 1936. Recherches sur l'activité rythmique des nerfs isolés. Paris: Hermann.
Foster, R. H. K. 1940. Proc. Soc. Exper. Biol. and Med., **44**:136.
Garrey, W. E. 1905. Amer. Jour Physiol., **13**:186.
Gray, J. 1922 Proc. Roy. Soc. London, B, **93**:122.
Gros, O. 1913. Arch. f. exper. Path. u. Pharmakol., **71**:395.
Guenther, A. E. 1905. Amer Jour. Physiol., **14**:73.
Heilbrunn, L. V. 1940. Physiol. Zoöl., **13**:88.
Heilbrunn, L. V., and Wilbur, K. M. 1937. Biol. Bull., **73**:557.
Higgins, S. G., and Fisher, D. 1924. Ann. Surg., **80**:268.
Howell, W. H. 1894. Jour. Physiol., **16**:476.
Howell, W. H., and Eaton, R. G. 1893. Jour Physiol., **14**:219.
Januschke, H. 1909. Arch. f. exper. Path. u Pharmakol., **61**:363.
Just, E. E. 1922. Science, **56**:202.
———. 1933. Zeitschr. f. Zellforsch u. mikr. Anat., **17**:25.
Koch, R 1881. Arch. f. exper. Path. u Pharmakol., **14**:153.
Locke, F. S. 1894 Jour. Physiol., **15**:119.
Loeb, J. 1901. Amer. Jour Physiol., **5**:362.
———. 1902. Pflüger's Arch. f. d. ges. Physiol., **93**:59.
MacCallum, J. B. 1903 Amer. Jour Physiol, **10**:101.
———. 1904. Univ. Calif. Publ. Physiol., **1**:115.
McCartney, E., and Ransom, F. 1917. Jour. Physiol., **51**:287.
Mathews, A. P. 1904. Amer. Jour Physiol, **11**:455.
Messini, M. 1929. Boll. Soc. ital biol. sper., **4**:418.
Moore, A. R. 1921. Proc. Soc. Exper. Biol. and Med., **18**:213.
Neuhof, H., and Hirshfeld, S. 1922. Ann. Surg., **76**:1.
Pasteels, J. J. 1935. Arch. de biol., **46**:229.
———. 1938. Trav. sta. zool. Wimereux, **13**:515.
Rebello, S., and Fontès, J 1925. Compt. rend. Soc. biol., **92**:909.
Robertson, T. B., and Burnett, T. C. 1912. Jour. Pharm. Exper. Therap., **3**:635.
Russo, G. 1935. Boll. Soc. ital. biol. sper., **10**:798.
Salant, W., and Hecht, S. 1915. Amer. Jour. Physiol., **36**:126.
Salant, W.; Kleitman, N.; and Wright, L. H. 1922. Amer. Jour. Physiol., **62**:531.
Salant, W.; Mitchell, C. W.; and Schwartze, E. W. 1916. Proc. Soc. Exper. Biol. and Med., **14**:16.
Salant, W., and Schwartze, E. W. 1917. Jour. Pharm., **9**:497.
Shafer, G. D. 1936. Jour. Pharm. Exper. Therap., **58**:264.
Shafer, G. D., and Crismon, J. M. 1936. Jour. Pharm. Exper. Therap., **58**:274.
Steinberg, A., and Brown, W. R. 1939. Amer. Jour. Physiol., **126**:638.
Sugimoto, T. 1913. Arch. f. exper. Path. u. Pharmakol., **74**:27.
Weil, R. 1915. Jour. Amer. Med. Assoc., **64**:425.
Wenner, W. F. 1931. Jour. Lab. Clin. Med., **16**:341.
Wilbur, K. M. 1939. Physiol. Zoöl., **12**:102.

PLATE I

FIG. 1.—15 per cent potassium citrate–sea-water mixture. Two polar bodies are seen beneath membrane.

FIG. 2.—Untreated egg in sea water. Intact germinal vesicle is indistinctly shown in center.

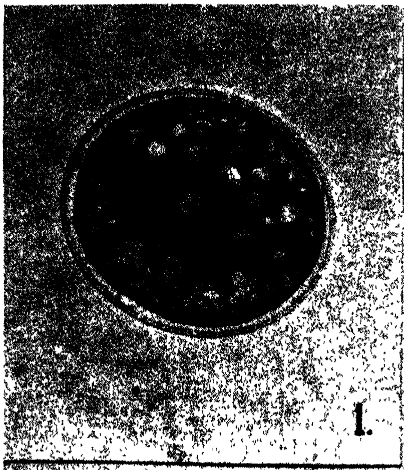
FIG. 3.—50 per cent potassium oxalate–sea-water mixture. Intact germinal vesicle is seen in the center of egg. Note membrane.

FIG. 4.—35 per cent potassium oxalate–sea-water mixture. Abnormal cleavage has taken place in some eggs.

FIG. 5.—25 per cent potassium oxalate–sea-water mixture. Abnormal cleavage is shown.

FIG. 6.—15 per cent potassium oxalate–sea-water mixture.

PLATE I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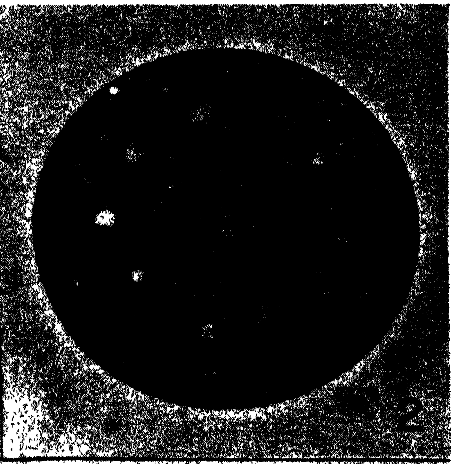
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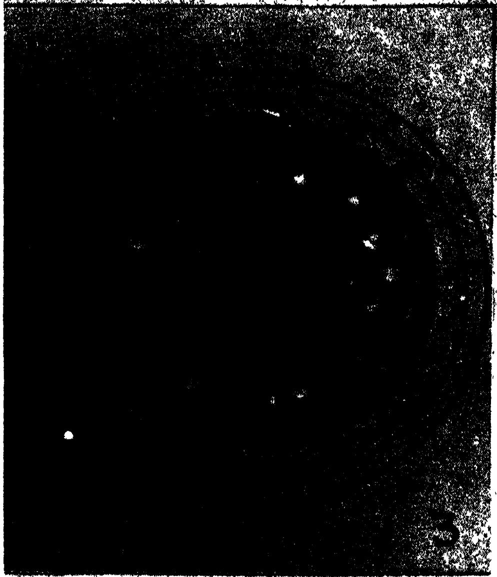
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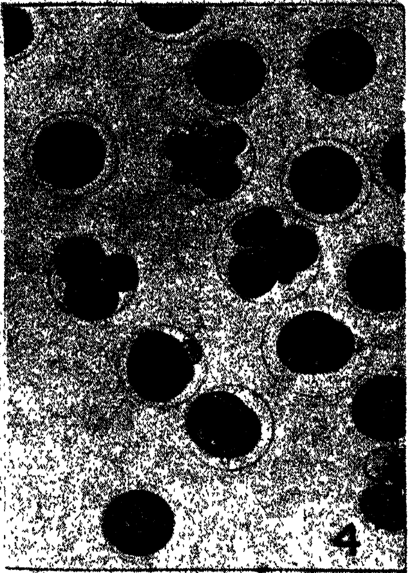
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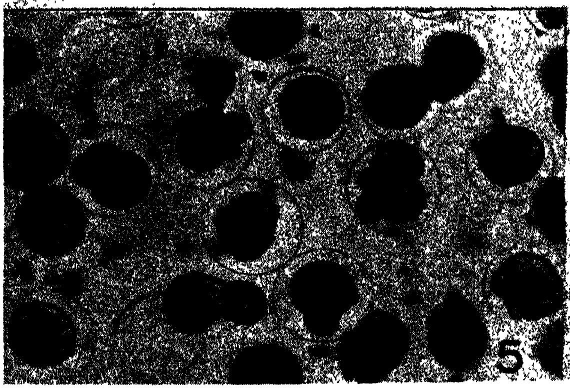
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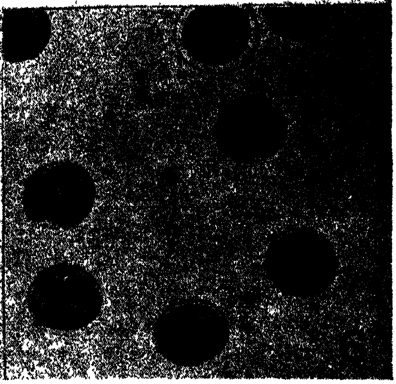
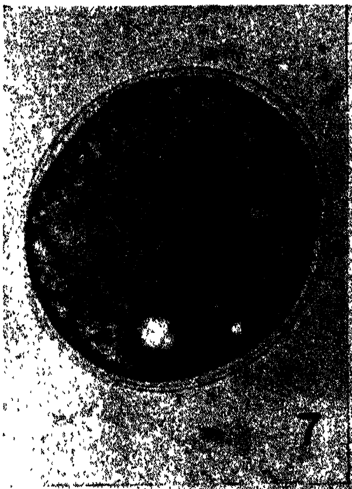


PLATE II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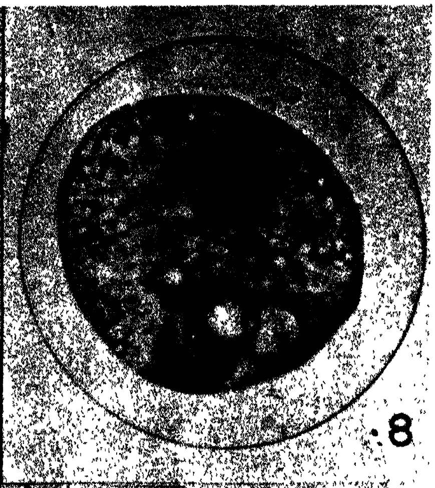
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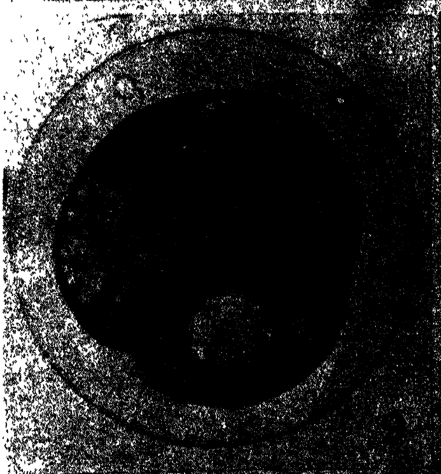

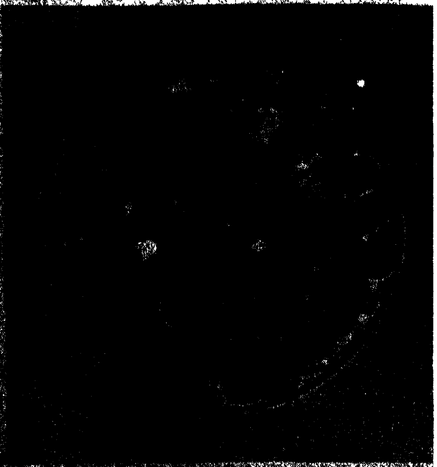

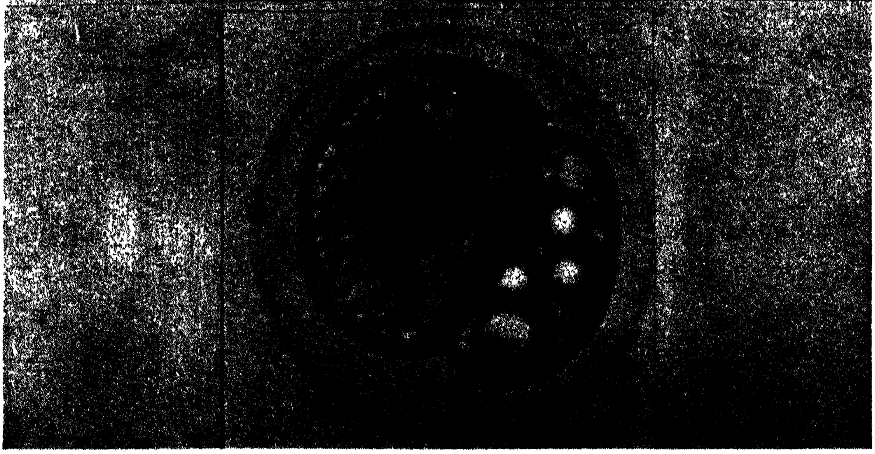

PLATE II

FIG. 7.—50 per cent KCl–sea-water mixture. Two polar bodies are present beneath membrane. Oil drops have moved to one side of the egg.

FIG. 8.—25 per cent sodium citrate–sea-water mixture. Two polar bodies are shown. Oil drops have coalesced.

FIG. 9.—25 per cent sodium citrate–sea-water mixture. Cleavage has taken place. Oil drops have coalesced forming 3 large drops. Two polar bodies have been given off. Pigmented area is seen near polar bodies.

FIG. 10.—25 per cent sodium citrate-sea-water mixture. Small portions of the egg have been budded off.

FIG. 11.—25 per cent sodium citrate–sea-water mixture. Cleavage has occurred. Coalescence of some of the original oil drops has occurred. Polar bodies are seen at the surface of larger blastomere. Pigment is concentrated in center of larger blastomere.

THE MELANOPHORE RESPONSES OF FISHES IN RELATION TO CONTRAST IN THE VISUAL FIELD[1]

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HOW does a fish adjust the shade of its integument to correspond with the background on which it is placed? It was in an attempt at a partial answer to this question that Sumner (1911) adapted to fish the "ratio hypothesis" of Keeble and Gamble (1904) which had been originally proposed to explain changes of shade in certain crustacea. According to this hypothesis, the factor which determines and makes possible the appropriate degree of integumentary pigment dispersion is the ratio of direct to reflected light which reaches the eyes of the animals, that is, the ratio between the light coming from above and the light reflected from surrounding objects. The very large value of this ratio on a black background produces maximal pigment dispersion; the very small value of the ratio on a white background results in maximal pigment concentration; and the particular value of the ratio on any background of intermediate shade determines the particular degree of pigment dispersion on that background. In other words, the greater the value of the ratio, the greater is the dispersion of pigment, and the darker is the shade of the fish.

Attempts to test this hypothesis have frequently yielded somewhat uncertain results. Sumner himself (1911) tried to obtain an abnormally low ratio of light above to light below by covering the vessels containing turbots and admitting light from below only but found that instead of becoming pale, as expected, most of the fish became somewhat dark. He believed that unwanted reflections played too large a part in his experiments. Mast (1914) from work with flounders concluded that "the interaction of the light received from the different immediate sources is probably not so simple as is demanded by the ratio hypothesis of Keeble and Gamble." Sumner and Keys (1929) made tests with two backgrounds on which the illumination was such that the ratio was in one case greater, in the other less, than that which would ordinarily exist. They found that the responses of *Hypsopsetta guttulata* agreed with the ratio idea but decided that "ratio" ought not to be quantitatively construed. Pearson (1930), with *Ameiurus*, repeated the work of Sumner and Keys and obtained similar results, but under other experimental conditions he did not find responses to be at all in conformity with the hypothesis. However, Brown (1936) measured the ratios existing on a series of black, gray, and white backgrounds and found that at intensities of 1.75 foot-candles or more the diameter of the melanin masses of *Ericymba buccata* varied directly with the ratio.

But, even though the truth of the hypothesis be granted, there may be a question as to how the ratio produces its effect. It may be that the response of the fish is a response to contrast as such; that the fish "sees" a contrast much as man does and makes adjustments accordingly. It may be, on the other hand, that the relationship between receptor and effector is a more direct one; that stimulation of one part of the retina leads directly to expansion, of another to contraction, of the melanophores. If so, the exact state of the melanophores will depend on the extent to which one or the other of these opposing stimuli predominates.

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[1] I am grateful to Dr. D. E. Minnich of the University of Minnesota for many favors received both while this work was in progress and at other times.

Investigators do seem to agree that adjustments of shade in fishes are made according to the relative stimulation of the upper and lower parts of the retina. Under natural conditions, of course, this would inevitably result from the position of the lighter sky above and the darker background below, but it appears that there may be a corresponding dorsoventral polarization and localization of function in the fish eye itself. Von Frisch (1911) found that covering either the entire eyes or the lower halves of the eyes of trout led to darkening but that covering the upper halves was without appreciable effect on changes of shade. Effects were, however, much greater when the lower halves only were covered than when the whole eyes were covered, and this was interpreted to mean that light reaching the upper halves was involved in the production of a contrast effect. His method of investigation was not entirely satisfactory, since the black paste used to cover the eyes frequently came loose or fell off in a very short time. An improved technique of the same type, consisting of the use of false corneas, or "goggles," was used by Sumner (1933). When the lower half of the visual field of *Fundulus parvipinnis* was darkened by this method, the fish became dark regardless of background. When the upper half was darkened, no visible change usually occurred on white, though a slight darkening might occur; but greater pallor than normal was seen when the fish were on a dark background. Darkening other parts of the visual field failed to give clear results, and attempts to rotate the eye in its socket without interfering with its function were unsuccessful. Sumner concluded from these experiments that the shade assumed by the fish was determined by the relative luminosity of the upper and lower parts of the visual field. Finally, Butcher (1938) found that

> covering the eye, rotating the eye, rotating the fish, destroying either the lower or upper region of the eye, and illumination from above or below on different backgrounds, all definitely show that the upper region of the retina of *Fundulus* is related to the pale response and the lower portion to the darkening of the body.

Nevertheless, I doubt that changes of shade in fishes can be wholly explained on the basis of the polarity of the eye. It is worthy of note that both in attempts to reduce the ratio of "direct/reflected" illumination to abnormally low values and in experiments which involved partial covering of the eyes a theoretically unexpected darkening of the fish has frequently appeared. Such darkening has commonly been interpreted, according to the nature of the experiment, as an effect of stray light reaching the eye by reflection or otherwise, or of light reaching receptors through the tissues of the head. In the following experiments with abnormally low ratios such darkening has been so marked that another explanation has seemed necessary.

EXPERIMENTS AND RESULTS

A number of experiments with an abnormally high proportion of light coming from below, similar to those originally performed by Sumner, were first made. A black wooden box, of dimensions to fit inside a small glass aquarium, 13 × 26 × 10 cm., was simply placed inside the aquarium containing the fish so as to shut off all light except from the bottom. To reduce the possibility of reflections at the upper surface of the water an opening in the top of the box, which could be closed by a blackened cork, allowed the escape of air; and the box was weighted so that no air space would remain above the water. Light of 10 foot-candles intensity, measured with a Weston illuminometer, was admitted through the bottom of the aquarium. Without appreciable difference in the results the light was sometimes transmitted through a sheet of opal glass under which a lamp was placed, sometimes reflected from a white enamel basin or from a sheet of white cardboard.

Under these conditions 46 individual tests, each preceded by adaptation for an hour to a black background under an illumination of 10 foot-candles, were made with a total of 15 specimens of the freshwater minnow, *Nocomis biguttatus* Kirtland. It was found that none of the fish remained maximally dark under the black box, but, on the other hand, none became pale. The fish in 15 tests were recorded as "slightly dark," indicating a considerable, but by no means maximal, blanching; in 31 tests as "moderately dark." If a low ratio of light in the upper part of the visual field to light in the lower part always produced contraction of the melanophores, these fish might have been expected to turn pale even more readily than on an ordinary white background.

In further tests the apparatus was changed, chiefly in order to provide greater ease of handling and observation. The darkened area in the visual field of the fish was somewhat reduced. The animals were placed in an 18-cm. crystallizing dish fitted with a floating black wooden lid, again provided with an opening for the escape of air. The depth of water used was just enough to allow the fish freedom of movement; however, they seldom moved except when first put into the dish. The dish rested on a glass plate, 75 × 75 cm., below which was supported a sheet of white cardboard. This apparatus was surrounded by sheets of opal glass, outside of which lamps were arranged on a level with the space between the cardboard and the glass plate. Adjustments were again made so that the intensity of the light reaching the bottom of the dish was about 10 foot-candles. This arrangement produced a "bottom" which appeared more clearly white than any that could be obtained with light transmitted directly from below through any available diffusing screen. Essentially, the fish were on a white background with a black area immediately above them.

Six of the fish which had previously been tested under the black box were adapted to a black background and tested again with the new equipment. Again, all remained somewhat dark. In each of 2 trials, 3 of the fish were found to become "slightly dark," while the other 3 were considered "moderately dark." As far as subjective impressions go, pigment dispersion appeared to be very little less than it had been when both the top and the sides of the container were dark. Removal of the black lid and the addition of light of about 20 foot-candles intensity from above quickly brought about a pronounced, persistent pallor.

The same 6 fish were also tested after being thoroughly blanched by adaptation to a white background for an hour. Under the black lid all 6 darkened slightly but definitely in each of 2 tests. Twenty-eight other fish taken at random were likewise individually adapted to a white background and then left under the black lid for ½ hour. Of these, 3 remained very pale, although there was some doubt as to whether they remained maximally so, 8 showed a very definite slight darkening, 14 became moderately dark, and 3 were considered "dark." It was quite clear that with the upper part of the visual field dark and the lower part light, most of these pale fish became darker.

The next step—again with the same 6 fish that had been carried through the preceding experimental variations—was to leave the apparatus as before except that the black lid was not used. Light then reached the fish from above by reflection from the white walls and ceiling of the room, the intensity of such light reaching the dish being 2 foot-candles. The light strength from below remained at 10 foot-candles. Under these conditions in each of 2 trials after previous adaptation to a black background 3 of the fish assumed a shade approaching an intermediate one, 2 blanched until they were only slightly dark, and 1 became very pale. The tendency toward melanophore contraction appeared to be somewhat greater than when the black cover was used, but except for the one fish it was still clearly incomplete. On the other hand, when the same fish were exposed to light of 2 foot-candles from above only, in the same dish, with the same cardboard under the glass as a background, approximately complete pallor appeared to result in all cases.

When the fish were first adapted to a white background and then exposed to 2 foot-candles illumination from above and 10 from below, again in each of 2 trials 1 darkened moderately, 4 darkened slightly, and 1 darkened slightly in one test but remained very pale in the other. No such darkening occurred when, over a white background, the fish were exposed to light of 2 foot-candles intensity from above only.

As a final and more critical test, when a shortage of *Nocomis* developed, 12 creek chubs (*Semotilus atromaculatus* Mitchill) were individually exposed to an illumination of 2 foot-candles intensity from above and 10 from below, with the same equipment as before. After 1 hour in this situation the fish were killed by being thrown into hot fixing fluid, and part of the integument was removed, mounted in glycerin, and examined. In each case a control animal, selected as closely as possible for similarity in size, appearance, and reaction to background, was used. This animal was immediately before or immediately afterward exposed to light of 2 foot-candles intensity from above only, in the same dish with the same cardboard as a background under the glass plate on which the dish rested. After an hour this animal was also killed, a piece of integument from the same part of the body was similarly prepared, and the two were compared under the microscope.

Strictly quantitative comparisons were not attempted, but the slides were examined not only by the experimenter but also by at least one unprejudiced observer, who without other knowledge of the experiment was asked to judge whether there was any difference in the condition of the melanophores on the two slides. In every case the melanophores were found to be more expanded in the fish which in addition to the light from above had received stronger illumination from below. Moreover, not only was pigment dispersion in a given individual greater than in its individual control, it was greater than in any control.

With few and somewhat doubtful exceptions, therefore, a partly expanded condition of the melanophores resulted whenever light reached the fish more strongly from below. Pigment dispersion appeared to be only slightly less when the top of the container only was darkened than when both the top and the sides were darkened; and some dispersion clearly occurred even when, in addition to the light from below, a weaker light was allowed to reach the fish from above.

DISCUSSION

It is clear that the total light intensity used in these experiments was more than sufficient to bring about or to maintain contraction of the melanophores. In some of the control experiments mentioned a much lower total intensity did bring about such contraction. Nor does it seem probable that the total intensity was too great to allow normal responses, for it was far less, for example, than that which would be returned to the eyes by a white background in bright daylight, where complete melanophore contraction occurs.

That undesired reflections played any considerable part in these experiments also appears improbable. No position could be found from which to the observer's eye the bottom of the vessel containing the fish appeared dark or the lower side of the cover appeared light. Moreover, no change in the behavior of fish exposed to light from above in either black or white dishes could be detected when the dishes were covered by one or more sheets of glass, even when the glass was not in contact with the water. With a diffuse-light source above, a fish in a black-bottomed glass dish covered by glass touching the water at every point must be in a situation very much like the one under the black cover as far as the incoming light and its reflections are concerned, except that the positions of the dark and light fields are interchanged. It would seem, therefore, that reflections can be ruled out and that the only significant effect of the black cover was to exclude light from above.

It has thus been shown that over a background on which melanophore contraction occurs under ordinary conditions a small ratio of light from above to light from below does not necessarily induce such contraction; that a very low ratio, with most of the light coming from below, may even bring about a partial melanophore expansion.

Evidently, therefore, a certain definite value of the ratio of "direct/reflected" illumination is required to bring about complete contraction of the melanophores. It might be suggested, then, that when the value of the ratio is very low, no background response occurs, just as none occurs when optic stimulation is completely absent. As a matter of fact, tests with 30 *Semotilus* did show that their appearance after an hour in the dark was very similar to their appearance after an hour under the black lid. The results in the dark were: 6 "pale," 12 "slightly dark," and 12 "moderately dark." Under the black cover the corresponding figures were: 5 "pale," 12 "slightly dark," and 13 "moderately dark." None were either maximally pale or maximally dark in either situation.

I believe that this signifies, not that the two situations are identical, but merely that the total activation of the melanophores is just enough to bring about a partly expanded condition in either case. In the dark, light forms no part of the total stimulation; in the light, the effects of light normally dominate all others. The difference between the two situations becomes clear from a consideration of the time required for melanophore change. In these particular trials there was no way of determining the exact time required for change in the dark. In other tests which have been made, however (cf. Danielson, 1938), the minimum time required to approach the equilibrium condition in darkness seemed to be at least ½ hour. Five or 10 minutes, as a rule, brought little or no change. The changes under the black cover, on the other hand, appeared to be completed in not more than 5 or 10 minutes. The two situations are, therefore, quite different, even though they result in about the same final appearance of the fish.

If we compare the foregoing experimental results from the use of very low ratios of "direct/reflected" light intensity—or, more properly, intensity above/intensity below—with the known results over various normally illuminated backgrounds, certain possibilities appear. Under an illumination of 10 foot-candles, a condition of 10/0 is normally approached, though it is not experimentally reached, over a black background. In the experiments just considered, the reciprocal of this, namely, 0/10, must have been approached when the fish were under the black covers. The result in the first instance is complete expansion of the melanophores; in the second, considerable, though incomplete, expansion. A ratio of 10/2 would be found over some gray background, while the ratio of 2/10 was established in one set of experiments. The condition of the melanophores over a gray background is one of partial expansion; and partial expansion, to a degree somewhat less than that obtained with a ratio of 0/10, also resulted when a ratio of 2/10 was used. The ratio reaching the fish eye over a white background illuminated from above could not be measured exactly because of shading by the instruments used, but it appeared to be between 10/6 and 10/8. There is certainly less difference between the numerator and the denominator over a normally illuminated white background than in any of the ratios previously mentioned. The effect of an ordinary white background is complete contraction (or no expansion) of the melanophores. The effect of either the ratio 0/10 or 2/10, therefore, resembles that of its reciprocal more than it resembles the effect of a normally illuminated white background. Moreover, melanophore expansion is greater with a ratio of 10/0 than with a ratio of 10/2; and in the same way, though perhaps less obviously, melanophore expansion is greater with a ratio of 0/10 than with a ratio of 2/10. The conclusion suggests itself that the ratio works either way, that the situation and the response are essentially similar whether the ratio is a/b or b/a.

It is true that the response to a situation in which more light comes from below differs in degree from the response to the more usual situation in which more light comes from above. Expansion of the melanophores is less under a black cover than over a black bottom. It is evident that the different parts of the retina are not completely interchangeable in the reception of the differences in illumination that form the stimuli for changes in shade; that a considerable specialization has occurred, either in different parts of the eye or in central nervous connections or both. It has been shown by Wunder in several papers (Wunder, 1933) and by Butcher (1938) that marked differences in the distribution of rods, cones, and retinal pigment between the upper and the lower parts of the fish retina commonly exist. It may be that because of such structural differences the fish "sees" a less vivid contrast when the conditions of illumination are the inverse of those naturally occurring. Specialization with respect to central connections is, of course, equally possible.

The fact remains that expansion of the melanophores in *Nocomis* and *Semotilus* occurs when the conditions of illumination are the inverse of those which normally evoke that response. Moreover, the same response to this situation, though unrecognized as such, seems to have been observed in other fishes. As previously mentioned, more or less melanophore expansion has frequently been described as a result of experiments involving either abnormal increases in the illumination from below or covering the upper part of the eye. All this indicates that the fish eye is much less strongly polarized than has been generally assumed. Although all parts of the eye are not equivalent, the state of the melanophores appears to be determined not by the stimulation of particular regions of the retina but by the degree of contrast in the visual field as a whole.

SUMMARY

Various partly expanded states of the melanophores of the freshwater minnows, *Nocomis biguttatus* and *Semotilus atromaculatus*, result over a well-illuminated white bottom when light from above is either excluded or relatively weak. Melanophore ad-

justments in a situation where more light reaches the fish from below than from above appear to be made according to the degree of contrast in the visual field in the same way, though not to the same extent, as over a comparable normally illuminated dark bottom.

LITERATURE CITED

BROWN, F. A., JR. 1936. Light intensity and melanophore response in the minnow, *Ericymba buccata* Cope. Biol. Bull., **70**:8–15.

BUTCHER, EARL O. 1938. The structure of the retina of *Fundulus heteroclitus* and the regions of the retina associated with the different chromatophoric responses. Jour. Exper. Zoöl., **79**:275–97.

DANIELSON, RAGNAR N. 1938. Light intensity and melanophore response in a cyprinid fish. Physiol. Zoöl., **11**:292–98.

FRISCH, KARL VON. 1911. Beiträge zur Physiologie der Pigmentzellen in der Fischhaut. Pflügers Arch. f. d. ges. Physiol., **138**:319–87.

KEEBLE, F. W., and GAMBLE, F. W. 1904. The colour-physiology of higher crustacea. Phil. Trans. Roy. Soc. London, B, **196**:295–388.

MAST, S. O. 1914. Changes in shade, color and pattern in fishes and their bearing on the problems of adaptation and behavior. Bull. U.S. Bur. Fish., **34**:177–238.

PEARSON, J. F. W. 1930. Changes in pigmentation exhibited by the freshwater catfish, *Ameiurus melas*, in response to differences in illumination. Ecology, **11**:703–12.

SUMNER, FRANCIS B. 1911. The adjustment of flatfishes to various backgrounds. Jour. Exper. Zoöl., **10**:409–505.

———. 1933. The differing effects of different parts of the visual field upon the chromatophore responses of fishes. Biol. Bull., **65**:266–82.

———, and KEYS, A. B. 1929. The effects of differences in the apparent source of illumination upon the shade assumed by a flatfish on a given background. Physiol. Zoöl., **2**:495–504.

WUNDER, W. 1933. Über den Bau der Netzhaut bei drei verschiedenen Formen der kleinen Maräne (*Coregonus albula* L.). Zeitschr. f. Morph. u. Ökol. d. Tiere (Zeitschr. f. wiss. Biol., Abt. A.), **27**:684–91.

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PHYSIOLOGY OF DEVELOPMENT OF THE FEATHER

V. EXPERIMENTAL MORPHOGENESIS[1]

(Five text figures; eight plates)

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I. INTRODUCTION

ALTHOUGH a great many studies have been presented on the physiology of development of feathers, none have dealt with the primary mechanism of development by methods of isolation of parts, their transplantation and recombination, such as are familiar in experimental embryology. Our own previous attempts to operate in such ways on the embryonic ring of cells known as the "collar" failed completely to yield positive results.[2] But, when we finally decided to operate directly on the papilla, which remains behind at the bottom of the follicle when the feather is plucked or naturally dropped in the process of molting, the first attempts yielded significant results, which were then extended by systematic experiments.

The operations have yielded a great number of abnormalities different from any hitherto recorded, and the types of abnormalities are definitely related to the types of operations. We are thus furnished with new material for a theory of development of feathers. As in experimental embryology, twins, "chimaerae," and many significant abnormalities and defects contribute to the theory.

Feathers of the breast tract of Brown Leghorn and White Leghorn fowl were used chiefly, but feathers from other tracts, especially from the saddle tract, were used occasionally for comparison and for special purposes such as the production of chimaera feathers.

II. THE NORMAL BREAST FEATHER: ANATOMY AND DEVELOPMENT OF THE RHACHIS

As most of the experiments deal with feathers of the breast and saddle tracts, photographs of representative feathers from these tracts are presented in Plate I (Fig. 6, *A* and *B*) as a basis for reference. It is also necessary to emphasize certain features both of the anatomy and of the development that have not hitherto been described or are not generally understood, in order to present the results of the experiments. These data are limited to the feathers of the breast tract.

1. ANATOMY OF THE NORMAL BREAST FEATHER

The feather is a difficult subject for dissection, and the incentive to tackle it, which we acquired from our experiments, has been lacking. Dissection of the feather requires a special technique, for the parts are so firmly bound together by tough keratin that dissecting instruments are otherwise of no avail. The method that we employed was immersion in 5 per cent KOH in aqueous solution; but this will not penetrate unless the external keratin is opened to permit access of KOH to the medulla of the rhachis. In order to do the least possible injury, a longitudinal incision is made along the ventral surface of the rhachis in the fluffy region; after this the feather is immersed in the KOH solution by a weight and allowed to remain in it from 8 to 12 hours. It is then washed in water and preserved in 70 per cent alcohol. The alkali passes along the entire length of the rhachis by diffusion through the medullary cells and softens the connections so that dissection becomes easy.

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[2] Attempts to operate directly on the collar of regenerating feathers *in situ* usually resulted in death of the feather but in any case yielded no positive results. Dr. Graham P. DuShane with our collaboration then made a very extensive series of transplants of portions of the collar to the chorio-allantoic membrane; these often yielded growth, but none of them contained any formed elements of the feather. The most conspicuous products were long fibers, presumably of keratin.

The feather to be dissected is placed in a large Petri dish flooded with 70 per cent alcohol. It is held at its apex by forceps while another pair of fine forceps in the operator's right hand gets a firm grip on some four or five barbs of one side of the vane close to their attachment to the rhachis and at a distance of about 1 cm. from the apex of the feather. With the feather thus caught in the two pairs of forceps, the barbs may be separated from the central part of the rhachis in a continuous strip by a gentle, steady movement. The forceps are moved back to new positions each time that a length covering some thirty or more barbs is detached. This can be continued to the calamus if so desired. The other side is similarly stripped off, leaving behind a relatively thick central rhachis with only a small tuft of barbs at its apex (Pl. II, Fig. 8).

Lillie and Juhn (1938) had concluded that the axis of the shaft is of entirely different embryological origin from lateral components formed from the bases of the barbs; but the anatomical consequences were not studied. The potash treatment makes this possible. Further dissection shows that the lateral, or ramic, components are composed exclusively of special modifications of the bases of the barbs, which we call the *barb petioles* (Pl. II, Fig. 9).

Each barb petiole (Pl. II, Fig. 10, *p.*) is a flattened plate curved to conform to the central rhachis; it forms a slight angle with the free axis of the barb and carries an extension of the series of proximal barbules (*p.r.*), about twelve to fourteen in number in the vane of a breast feather, as far as the insertion of the next proximal barb; these barbules, which appear to arise from the shaft between the insertions of barbs, thus belong primarily to the latter. Long keratin fibers (*f.*) arising within the petioles extend a distance of two or more barb intervals, thus building up a continuous fibrous base of the lateral components of the rhachis.

The medullary cells of the barbs terminate above the bases of the petioles (Pl. II, Fig. 10, arrow) and are thus not continuous with the medullary cells of the central rhachis. It is an outstanding feature of the petioles forming the ramic components of the rhachis that they are entirely devoid of medullary cells.

However, at the very apex of the feather the medullary cells of the two apical barbs, one on each side, are continuous with one another and with those of the rhachis. The same relation may be found in the second barb on each side, though the connection is constricted; the connection is lost in the third barb, though we do not assert that this is exactly so in all specimens. It is about in this region, then, that we place the apical termination of the central rhachis, the medullary cells of which are continuous with those of the first two barbs on each side. This anatomical determination is confirmed by study of the development.

The central rhachis itself shows evidence of bilateral structure in the ventral groove and the heart-shaped form of the medulla in cross-section; quite frequently in our experimental feathers, and once in a natural breast feather, we have observed complete lateral doubling of the medullary cells involving part or all of the length of the rhachis. The central rhachis arises embryologically from a spreading base in the collar extended laterally, and the disposition and orientation of the melanophores in the growing base indicate a bilateral origin for those which enter the rhachis (cf. Lillie and Juhn [1938], p. 440 and Figs. 7 and 17). Finally, we have numerous (twenty-one) cases in the experiments recorded in this paper in which only one lateral half of the rhachis is formed. We therefore regard the rhachis as composed of two lateral halves, in each of which we recognize a central component and a lateral (ramic) component.

2. THE DEVELOPMENT OF THE RHACHIS

The rhachis does not begin to arise until after a large number of apical barbs have formed. On the sixth day of regeneration about fifty of approximately equal length are found in the dorsal part of the germ as a continuous series occupying about two-thirds of the circumference. Their orientation is strictly axial, and they accordingly lie parallel to one another and to the long axis of the cylinder (Fig. 1, *a;* cf. Lillie and Juhn, 1932). They are then added to from both sides of the ventral field progressively and very rapidly toward its center by new barb apexes until most of the field is occupied. During the sixth day tangential movements of the barbs are absent.

After the dorsal barbs have reached a length of about 2 mm. (on or about the seventh day), two to four of them in the center of the dorsal surface fuse at their bases, and the central rhachis arises in the collar immediately beneath them, fusing with the conjoined base (Fig. 1, *b* and *c*). At this time tangential movements of the barbs begin at their junction with the collar, producing a bend toward the rhachis in the case of the bases of the original barbs; as this movement extends ventrally, the barbs begin to describe spiral courses around half of the cylinder on each side. The most ventral ones thus define in the center of the ventral field a ventral triangle from which the tangential movements are directed to the right and to the left. As the central rhachis grows axially, the series of forming barbs moving toward it become united to it by their forming petioles.

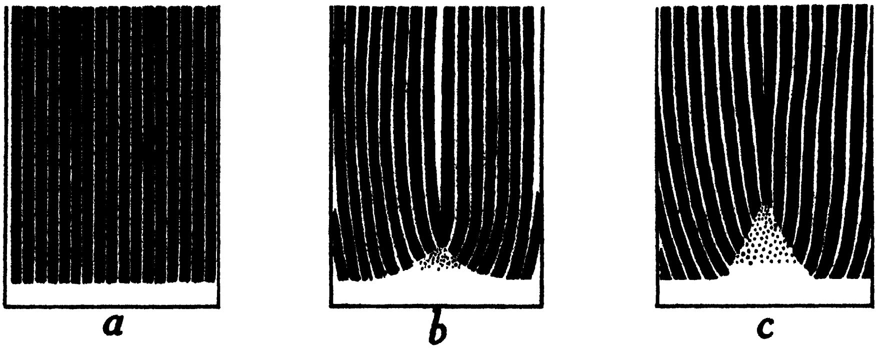

FIG. 1.—Diagrams of the bases of barbs in the center of the dorsal surface of feather germs of a Brown Leghorn capon: *a*, of 6 days' regeneration, *b*, of 7 days', *c*, of 8 days'. The origin of the central rhachis (stippled) is shown in *b* and *c*. The diagrams are based on cleared mounts of entire feather germs. The framed space below the barbs represents the margin of the collar (cf. the text).

Barb petioles constituting the lateral components of the rhachis are not formed in the case of the two apical barbs and occasionally of one or two more, which fused at their bases before the central rhachis began to form. This agrees with the anatomical facts already alluded to (p. 105). Barb petioles, and hence lateral components of the rhachis, are formed from this point proximally.

III. THE STRUCTURE AND FUNCTIONS OF THE PAPILLA

The term "papilla" has been used in diverse ways. In early studies on development of the feather no distinction was made between "papilla" and "pulp." The germ for replacement and regeneration has also sometimes been regarded as something formed anew by the corium for each occasion (Woitkewitsch, 1936). It is therefore necessary to emphasize our view that, while the pulp is formed anew for each replacement of a feather, the *papilla* is a persistent structure from the time of its embryonic origin through all successive generations of feathers.

In all our numerous experiments no follicle from which the papilla has been removed has ever regenerated a feather; and, on the other hand, an isolated papilla implanted subdermally under favorable conditions regenerates both feather and follicle (cf. p. 112).

The papilla has two main functions—first, to produce the vascular pulp concerned in the nutrition of the growing feather, which was dealt with fully in the preceding paper of this series (Lillie, 1940), and, second, the morphogenetic function, with which the present paper deals, to which reference was also made in the preceding paper (p. 145).

The relations of the papilla to the feather cylinder[3] during regeneration are shown in Figure 2 and in more detail in Figure 3. The papilla is embraced and constricted by the margin of the collar (inferior umbilicus). The distal portion above the umbilicus is continuous with the pulp; and the basal portion below the umbilicus, shaped like an inverted mushroom, is firmly anchored by its margins in the wall of the follicle. The broken line in Figure 3 represents approximately the boundary between the pulp-forming part of the papilla and the part concerned with control of morphogenesis. The papilla of a feather in process of regeneration will be called an "active papilla."

Figure 11 (Pl. III) represents an axial section of an active papilla immediately after

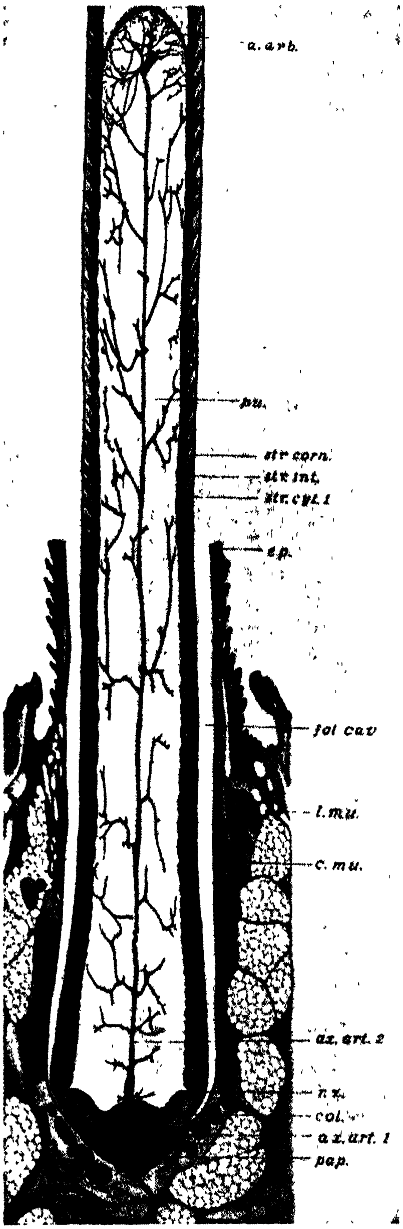

FIG. 2.—Longitudinal section of the feather cylinder and follicle on the twenty-first day of regeneration, White Leghorn cock. Semi-diagrammatic. It shows the arterial circulation in the pulp in addition to the features mentioned in the text (from Lillie, 1940). ×18.

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[3] We adopt this excellent term from 'Espinasse (1940); it includes the developing and growing epidermal part of the regenerating feather held together by the sheath in cylindrical form. In our sense it refers more specifically to the part containing the pulp. It is not a complete substitute for the common term "feather germ," which is a generalized and indefinite term similar to "embryo."

the feather has been plucked.[4] The constriction, of course, represents the position of the umbilical lip of the collar (cf. Fig. 3). The torn apical surface devoid of ectoderm is through the plane of breakage due to plucking about in the position of the broken line in Figure 3. A thin epidermal layer covers all the remainder of the papilla, beginning abruptly at the sides of the torn apex.

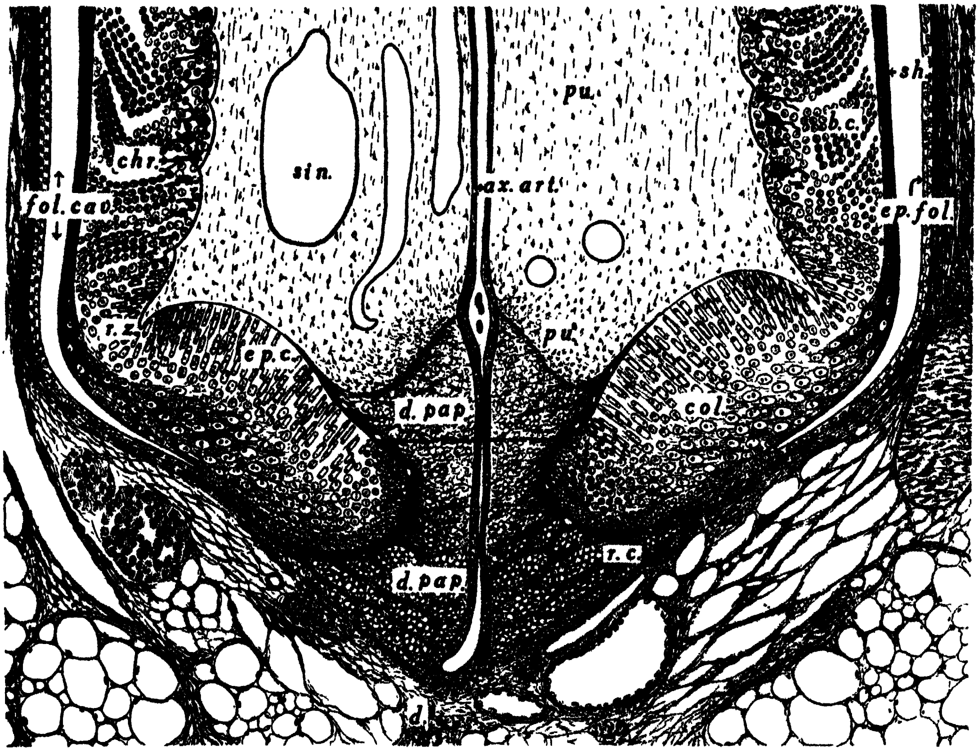

FIG. 3.—Diagram of the region of the collar and papilla corresponding to about the twenty-first day of regeneration. The relative width of the collar and the epidermal cylinder is exaggerated in order to bring out details of cells. The broken horizontal line represents the plane of rupture across the papilla when the regenerating feather is plucked. *ax. art.* = axial artery; *b.c.* = barbule cells; *chr.* = chromatophores; *col.* = collar; *d.* = derma; *d. pap.* = dermal papilla; *ep. c* = columnar epithelium of collar with elongated nuclei; *ep.fol.* = epidermis lining wall of follicle; *fol. cav.* = cavity of follicle; *sin.* = blood sinus; *pu.* = pulp; *pu'.* = transition zone between pulp and papilla; *r.c.* = regeneration cells; *r.z.* = ramogenous zone; *sh.* = sheath.

The derivation of the epidermal cells is from the regeneration cells of the collar and the wall of the follicle (Fig. 3, *r.c.*). They extend on both sides of the umbilicus as far as the boundaries of the papilla, thus, on the side of the pulp to the junction of the papilla with the pulp and on the opposite side likewise to the margin of the papilla, where they gradually thin out into the epidermal lining of the follicle. On the pulp side the regeneration cells are sharply bounded from the specialized cylindrical cells of the collar by a notch. When the feather is plucked, the regeneration cells are torn from the collar and remain attached to the mesodermal papilla, so intimate is their union, by means of a thick, structureless, basement membrane. Correspondingly, sections of the plucked feather itself through the collar show complete absence of regeneration cells over and on both sides of the umbilical lip.

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[4] Fifteen active and ten resting papillae have been studied in serial sections in order to satisfy ourselves concerning their uniformity.

The regeneration cells, which form the ectoderm of the papilla, are totipotent as far as the feather is concerned; by their growth, multiplication, and differentiation they produce a new feather complete in all its parts, under control of certain morphogenetic functions of the dermal papilla.

After regeneration is completed, the papilla lies beneath the inferior umbilicus of the feather in a resting state. The mesoderm of the resting papilla (Pl. III, Fig. 12) consists of a more cellular (basophilic) apical half and a more fibrous (acidophilic) basal half. Between the apical and the basal halves there is an ill-defined peripheral constriction. The surface of the thick mesodermal core is covered by a thin, single-layered epidermis which may, however, be deficient over the apex.

IV. EXPERIMENTS ON THE PAPILLA

1. TECHNIQUE

The following account relates primarily to feathers from the anterior portion of the breast which were used in a great majority of the experiments. Operations on feather papillae from other tracts differ only in detail. The anterior breast is an advantageous region for the operations because of its ready accessibility and because the follicles are oriented obliquely in the skin and there is relatively little fat surrounding them.

Most of the operations have been made on the active papillae of regenerating feathers from 15 to 30 days of age. Such an active papilla is larger than the resting papilla of the finished feather, and the wall of the follicle is thinner and more flaccid—both conditions that facilitate operation. However, a smaller number of operations made on resting papillae yielded results similar in principle, so that we are convinced that the resting papilla as a morphogenetic system differs in no essential way from the active papilla.

The bird to be operated on is fastened to an operating board by loose knots around the base of the wings and on both feet. It is anesthetized before operation by intraperitoneal injection of Nembutal (pentobarbital sodium, Abbott) with a dosage of about 0.5 cc. for each kilogram body weight, which puts the bird to sleep for 2 or 3 hours.

The region around the feathers selected for operation is then plucked, or the feathers surrounding the selected ones are clipped, and the bird is covered with clean, dry towels except for the operation site which is washed with 70 per cent alcohol. The feathers selected for the operations are plucked immediately before operating.

Instruments used in the operation are first washed in hot water, then left on a clean towel, and immediately before use are sterilized in 70 per cent alcohol For the purpose of washing away blood and moistening tissues during operations, sterile Ringer's solution is used.

Operations are made under a binocular microscope arranged so that it can be swung over the site of operation. When the selected follicle is in focus, a longitudinal incision is made along its upper surface to a point near the bottom of the follicle, and the flaps are reflected. This should be done with a single clean cut.

The actual cuts on the papillae are made with the finest iridectomy scissors. With two pairs of very fine steel forceps the papilla is brought into view by grasping the wall of the follicle near the bottom and gently lifting the papilla. The iridectomy scissors are then taken up, and the operation on the papilla is made by a single cut, or as few cuts as are required to remove the parts indicated in the diagrams of operations (Fig. 4). The birds stand the operations well. Deaths of experimental birds referred to in the text were due to extraneous causes.

Operations of Type A, involving only the apical half of the papilla, are quite readily performed, because the apical half of the papilla protrudes freely into the follicular cavity and there is a distinct difference in the degree of opacity of the apical and the basal halves of the papilla. In operations of Type B, involving the entire length of the papilla, the cuts must carry through the bottom of the follicle.

It is easy to remove the entire papilla, or definite parts of it, for transplantation or recombination experiments. In such experiments part of the follicular wall is left as a means of ready handling. The papilla may be left in Ringer's solution while the transplantation site is being prepared.

We have not attempted to use marks on the skin by tattooing or otherwise for identification of papillae. The practice has been to map the positions of the operated follicles with reference to established landmarks on the bird's breast, such as clipped feathers immediately surrounding the operated ones. The fact that all operated papillae are relatively slow in beginning regeneration may be used to differentiate them from others of the same age. Moreover, the follicles of operated papillae may be distinguished by the scar along the line of healing, so that proper identification offers no difficulty.

2. INTRODUCTION TO EXPERIMENTS

All operations on the papilla are oriented as exactly as possible. Inasmuch as the papilla exhibits no structural differences except along the primary axis, the dorsoventral orientation is determined *in situ* while the operations are being performed by the relations of the papilla in the follicle to the dorsal and ventral surfaces of the feather. We can recognize *in situ* apical and basal ends, dorsal and ventral surfaces, and right and left sides of the papilla; operations can be made accordingly in transverse, sagittal, and frontal planes at selected levels. But the absence of landmarks creates a difficulty in precise localization of cuts.

Without exception, feathers derived from operated papillae are slow in emerging from the mouth of the follicle in comparison with those derived from normal papillae. The repair of the follicle, which always takes place very rapidly after the operation, does not seem to be a considerable factor in the delay. Factors inherent in the operated papilla itself seem to us more important. Among these are the effective restoration of the blood circulation, repair of the ectoderm on the wounded surface, and, presumably, certain obscure adjustments of the mesoderm of the papilla. The time of emergence of feathers derived from normal papillae is from 7 to 9 days in the breast tract. This time is increased in general to 13 to 15 days in the case of operated papillae, but in the case of removal of the apical half of the papilla (Operation *T*, Fig. 4) the time of emergence is still longer, at least 18 days.[5]

After the feather has emerged from the follicle its rate of axial growth may be approximately normal, and the length attained may be equal to that of control feathers. This happens only in case a normal rhachis develops, as, for instance, in feathers derived from dorsal halves of the papilla; but in other cases the rate of axial growth and the length attained are diminished, sometimes to an extreme extent.

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[5] Over half of the total number of operations, which exceed five hundred in number, have yielded feathers of some sort and are in that sense successful. The percentage is, of course, lower in some operations than in others.

The types of experiments are similar to those in experimental embryology: (1) "transplantation experiments," in which a papilla or a part is transplanted to a new environment, e.g., to another follicle, or subdermally outside of a feather tract; (2) "defect experiments," in which a determinate part is removed and discarded and the remainder allowed to develop; (3) "isolation experiments," in which the papilla is merely divided in a determinate way and the parts left to develop; and (4) "recombination experiments," in which parts of two papillae are brought together in the same follicle.

3. TRANSPLANTATION EXPERIMENTS: ORIENTATION AND TRACT SPECIFICITY OF PAPILLAE

Transplants of isolated papillae have been made for the most part to sites on the same individual (autoplastic); the same technique may be used between different individuals of the same breed or between individuals of different breeds, but these do not succeed so readily; they also introduce other problems that are not considered in this paper.

Autotransplants have been made within the same follicle, to follicles of other tracts from which their own papillae were removed, and to subdermal locations between feather tracts. Within the same follicle the "transplantation" is merely a rotation of the papilla around its own axis, after it has been dissected completely free, through 90° or 180°. The operation is for the purpose of determining from the orientation of the resulting feather whether or not the normal dorsoventral orientation of the feather is due to an inherent dorsoventral organization of the papilla.

In a particular experiment on a Brown Leghorn hen, after plucking the feathers, six follicles were opened along their length to near the bottom; in each the papilla was then dissected entirely free from all attachments, rotated either 180° or 90° along its axis, and then replaced. Two failed to develop (Nos. 2 and 3). Of those that developed, the ventral surface was up and the dorsal surface next to the skin in two (Nos. 4 and 6) that were rotated 180° on the long axis; in the two others, in which the papillae were rotated 90°, the feathers also were rotated about 90°.

In another experiment, on a Brown Leghorn male, that involved also the question of tract specificity of isolated papillae, seven papillae of regenerating saddle feathers were grafted into breast follicles from which the papillae had been removed, three in normal orientation and four rotated 180° axially. One of the former formed a saddle feather with normal orientation, while two failed to develop. Two of the four with reversed orientation developed saddle feathers ventral side up, while the other two failed to develop. Similarly, breast papillae transplanted to saddle follicles developed into breast feathers (three out of five cases).

Breast papillae dissected out of the follicle and free from other tissue have been transplanted subdermally in the region of naked skin between breast and dorsal feather tracts that is protected against external injury by the wing in its natural position. Subdermal blood vessels can be seen through the thin skin of this region, and a location at the angle of a branch is selected as favorable for vascularization of the implant.

Regeneration is always unusually slow in the case of such implants. This is presumed to be due to the mechanical difficulties of piercing through the skin, which happened only once spontaneously; usually the graft becomes encapsulated in connective tissue, but evidences of growth are obvious by subdermal swellings. In three cases the struggling germs were liberated by cutting through the skin, and they developed into external feathers.

From eighteen such implants of breast papillae four have developed breast feathers, which have grown through the skin and formed typical follicles. In one case the bird died before a second-generation feather could be formed; another bird died after the second-generation feather was well developed. The third bird had two successful grafts, in their third generation at the time of writing. The transplanted papillae have therefore become established. The feathers are disoriented dorsoventrally and with reference to the axis of the body, owing to the fact that fixed orientation cannot be given to the transplanted papillae.

The transplantation experiments demonstrate that the axial and the dorsoventral orientation of the feather is determined by intrinsic functions of the papilla and also that the papilla carries with it the specificities of the tract of origin.

Experiments on transplantation of skin from one feather tract to another have shown that the transplants develop feathers of the type of the tract of origin (Danforth, 1929, 1937). The present experiments show that the tract specificity of the feather resides in the individual papillae, not in the transplant as a whole. Transplantation of isolated papillae is applicable to more exact problems, as, for instance, the production of chimaera feathers (p. 118) or interbreed reactions of melanophores where the papilla is of foreign origin.

4. THE DEFECT EXPERIMENTS

A. INTRODUCTION

Defect experiments are the fundamental tests for the morphogenetic properties of the papilla. Several principal kinds have been carried out, as shown in the diagram of operations (Fig. 4). They will be designated as follows:

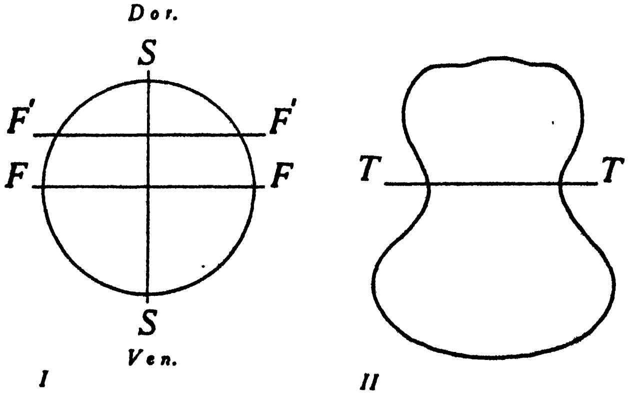

FIG. 4.—Diagram of operations. I. Cross-section of the papilla to show planes of operations F and F′, equal and unequal frontal operations, and S, sagittal. II. Side view of the papilla to show the position of the transverse operation T.

T (transverse).—The papilla is bisected transversely, and the apical portion discarded. The experiment tests the developmental capacity of the basal portion of the papilla. Only one level of amputation has been tested, approximately halfway between apical and basal surfaces of the papilla.

F (frontal).—The plane of operation is frontal. The F experiments through the center of the papilla are of two kinds, A and B; in the A type of experiment the operations are limited to the apical half of the papilla (F.A.); in the B type the operations involve the entire papilla (F.B.) and thus also a certain minimal amount of the bottom of the follicle. Each of these again presents two alternatives according as whether the dorsal (F.A.D. or F.B.D.) or the ventral part (F.A.V. or F.B.V.) is discarded. The *F′* plane was used only to determine the developmental capacity of the dorsal quarter of the papilla (F′.B.V.).

S (*sagittal*).—The plane of operation is sagittal through the center of the papilla. Here also A (S.A.) and B (S.B.) types of operation are distinguished. Either right or left parts may be discarded.

B. CLASSIFICATION AND DESCRIPTION OF ABNORMALITIES

From the defect experiments alone we have preserved and filed about one hundred and fifty feathers belonging to the first generation after operation. These present an astonishing array of abnormal feather forms, no two of which are precisely alike. All, however, are composed, so to speak, of certain "elementary abnormalities" of the rhachis and barbs. The same elements of abnormal structure also occur in isolation and recombination experiments. They may be classified as follows.

I. Elementary abnormalities of the rhachis
  1. *Absent.*—Absence of the rhachis may be of any degree from apex to base; it never occurs in an intermediate position. A "tuft feather" results (Pl. IV, Figs. 18 and 19; Pl. VIII, Fig. 28).
  2. *Halved.*—One lateral half of the rhachis is missing,[6] a condition that likewise occurs in variable extents from apex to base and not in intermediate positions. A half-vane type of feather results (Pl. V, Figs. 20 and 21).

II. Principal elementary abnormalities of the barbs
  1. *Branched.*—One or more dichotomous divisions of a single barb base (Pl. III, Figs. 13 and 14, *et passim*).
  2. *United.*—Two or more barb bases united apically (Pl. VII, Fig. 26).
  3. *Bundled.*[7]—A complex of barbs with apical branching from an intermediate union of separate barb bases (Pl. VII, Figs. 25 and 26; Pl. VIII, Fig. 29).
  4. *Reversed.*—A barb attached by its apex to a half-rhachis on the side opposite to the vane (Pl. V, Figs. 20 and 21).

Barbs also exhibit many other abnormalities not included in this list. For instance, the serial order on the rhachis of two barbs may be reversed at the margin of the feather so that the barbs cross at some point but are otherwise interlocked like normal barbs (see Pl. VII, Fig. 25, left side). This phenomenon is common in natural (Section IV, 9), as it is also in experimental, feathers. Occasionally the crossing may involve three or even four barbs. Crossed barbs are frequently, perhaps always, bent. In the experimental feathers barbs may be stubby and much shorter than their neighbors or attenuated and much longer; they may be of much more, or much less, than normal diameter. Examples may be seen in the photographs. Abnormalities of barbules also occur.

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[6] For this interpretation strictly anatomical criteria pertaining to the rhachis itself are uncertain. The designation "halved" is derived from the organization of barbs on one side only into a typical half-vane and the lack of any such organization of barbs on the other. It is essentially a functional interpretation.

[7] The term "bundled" is used in this paper to denote only cases in which there is actual fusion of the stems of the barbs concerned to such an extent that the individuality of the component elements is apparently lost in the common section. Theoretically, however, there may be all degrees of approach to this condition from mere contact of barb stems through stages of partial fusion. Such cases are certainly extremely rare in the experiments that form the subject of this paper; but we have found numerous examples along fault bars in certain unpublished experiments of a different kind. The number of apexes concerned in such cases always agrees with the number of bases and the individuality of the barbs is not lost (cf. p. 125).

We shall, however, lay emphasis on the four principal elementary abnormalities of the barbs listed above. These are correlated with abnormalities of the rhachis. When the rhachis is absent, the barbs develop in the form of a tuft in which it is difficult to recognize any bilateral distribution (Pl. VIII, Fig. 28; Pl. IV, Figs. 18 and 19). Such feathers develop in the wall of a cylinder containing pulp, as in normal feathers, and complete their growth with resorption of pulp over a longer period of time than normal. The number of barbs produced is, however, much less than in a corresponding normal feather; counting all apexes, the total number is about one hundred and twenty-five in the specimen shown in Figure 18 (Pl. IV), as compared with an average of five hundred and forty in ten normal breast feathers of the Brown Leghorn capon. The barbs may attain excessive lengths, and, when growth is completed, no barb is of the pure vane type, but all have fluffy bases or are completely fluffy. From this it is clear that any barb will change from vane-type to fluff-type if its period of development is sufficiently prolonged and, by corollary, that union with the rhachis is required to terminate the growth of any barb, which otherwise is unlimited within the period of development of the feather. We interpret the tuft feather as one in which growth of the barbs has been indefinitely continued in a strictly axial direction.

In Figure 28 (Pl. VIII) the rhachis is present in the cylindrical part of the feather; in Figures 18 and 19 (Pl. IV) the rhachis is certainly much diminished, perhaps entirely absent in Figure 19, although the calamus is formed.

If one lateral half of the rhachis is missing, as in Figures 20 and 21 (Pl. V), the barbs are abnormal on the missing side and normal on the opposite side, according to the length of the half-rhachis, thus producing the "half-vane" type of feather, of which we have numerous examples. The barbs of the defective side are similar to those of feathers lacking the rhachis entirely, except that those next to the half-rhachis are usually of the extraordinary type of "reversed" barbs, i.e., attached by their apexes to the half-rhachis. Reversed barbs may have free bases, or the bases may be in the feather cylinder or attached to the same half-rhachis ("looped," Pl. III, Fig. 17).

Operations involving the apical half of the papilla alone usually produce feathers with "apical defects," limited to between 1 and 2 cm., the remainder of the feather being normal. Examples are shown in Plate III (Figs. 13–17), and in Plate VII (Fig. 26). All classes of barb abnormalities may be found in the defective region; they are more pronounced on one side than on the other, which may, indeed, bear only normal barbs as in Figures 15 and 16. The correlated abnormality of the rhachis resembles the type of halving rather than of absence, even to the extent of occasional presence of reversed barbs (Fig. 17).

We have thus distinguished three principal types of abnormal feathers—tuft feathers, half-vane feathers, and those with apical defects only. There are also occasional specimens not readily classifiable under these types, including several veritable "museum pieces." A segment of one of the latter is shown in Plate VII (Fig. 27). Such cases also are composed of the same elements of abnormal structure as the principal types.

C. RELATION BETWEEN TYPES OF OPERATION AND RESULTS

In this section we shall correlate the types of abnormal feathers produced in the defect experiments with the main types of operation (Fig. 4).

1. *Experiment T.*—The apical half of the papilla is amputated. The location of the cut is approximately in the center of the constriction corresponding to the lip of the umbilicus (Fig. 4). The amount of the papilla removed is subject to slight fluctuations.

Nine feathers of the first generation resulted from this experiment on active papillae. Seven of these have apical defects only, as illustrated in Plate III (Fig. 15) for one case; the defective region in this case is 10 mm. long, or about 12 per cent of the total length of the feather; in the other six cases of apical defects derived from this experiment the elementary abnormalities differ, and the length of the defective region varies from 5 to 11 mm. Thus it becomes evident that the basal half of the papilla is capable of producing about 90 per cent of the length of the feather in normal fashion.

Of the two exceptional feathers derived from this experiment, one was dwarfed but essentially normal in structure; the other also was dwarfed (only 5 cm. long) and highly abnormal on one side (Pl. VII, Fig. 27).

The same experiment on resting papillae yielded seven feathers, of which five showed apical defects of varying extent, and two were approximately normal.

2. *Experiments F and F'.*—There are five varieties of defect operations in the frontal plane: F.A.V., F.A.D., F.B.V., F'.B.V., and F.B.D.

a) *F.A.V.*—The ventral half of the apical half of the papilla is removed. We have five first-generation feathers resulting from this operation, of which three are normal feathers except that the apexes are narrower than in the controls and slightly bent laterally; one has very minor apical defects; the last has greater apical defects.

If these results be compared with the T experiments, agreement will be noted in limitation of defects to the apex of the feather, but normal apexes also occur in the F.A.V. experiment in three out of five cases. This suggests that the ventral part of the papilla is not needed for the normal development of the rhachis.

b) *F.A.D.*—The dorsal half of the apical half of the papilla is removed. We have twenty-six first generation feathers resulting from this experiment. Three of these are nearly normal throughout, sixteen have apical defects only, and the remaining seven are abnormal throughout like F.B.D. experiments. The group of sixteen resemble the T experiments both in having defects limited to the apex and also in the defective characters of rhachis and barbs, from which we may conclude provisionally that the dorsal surface of the apical half of the papilla is necessary for normal development of the rhachis. The remaining seven with defects along the entire length of the feather are to be explained by removal of a greater portion of the dorsal surface of the papilla than intended (cf. F.B.D.).

c) *F.B.V.*—The entire ventral half of the papilla is removed, and the dorsal half develops alone. We have four first-generation feathers derived from this experiment on active papillae, of which three are approximately normal; in the fourth the vane portion is abnormal, carrying two or three reversed barbs, but the region of the fluff is normal. The normals were strong, growing feathers with rate of growth approximately equal to the control. The aberrant feather can be explained by injury to the dorsal surface in the operation. From the same experiment on resting papillae five feathers with normal shafts were derived, and one dwarf vane. We may thus conclude that the dorsal half of the papilla is capable of producing normal feathers.

d) *F′.B.V.*—Three-quarters of the ventral portion of the papilla is removed, and the dorsal quarter develops alone (Fig. 4, F′).

This experiment was made for the purpose of locating more precisely the primordium of the rhachis in the papilla. We have ten first-generation feathers resulting from this experiment on active papillae. Six of these are nearly normal, one shows typical apical defects, and three are highly abnormal. The dorsal quarter, like the dorsal half, produces strong growing feathers, with the exception of the highly abnormal minority. The ventral three-quarters of the papilla is not necessary for the development of a normal rhachis.

e) *F.B.D.*—The dorsal half of the papilla is removed, and the ventral half develops alone.

The development of the ventral half of the papilla offers the greatest contrast to that of the dorsal half. We have thirteen first-generation feathers derived from this experiment on active papillae; four of these are tuft feathers lacking the rhachis entirely, six are of the half-vane type (Pl. V, Fig. 20), two have a rather long, defective apical region resembling the F.A.D. type, and the last was a much delayed dwarf feather of no regular type. Thus the characteristic result of this experiment is absence of the rhachis or its development on one side only, always reduced in length.

From the same experiment on resting papillae six tuft feathers of varying degrees (cf. Pl. IV, Fig. 19, and Pl. VIII, Fig. 28), three half-vane feathers and one feather with apical defects were derived. There was thus a larger proportion of tuft compared with half-vane feathers than in this experiment on active papillae.

3. *Experiment S.*—There are three types of defect operations in the sagittal plane; in one (S.A.) the attempt was made to remove only a lateral half of the apical half of the papilla; in the second (S.B.) a lateral half of the entire papilla was removed; in the last, sagittal thirds were tested.

a) *S.A.*—We have five feathers resulting from this experiment; three of them are normal or with very minor apical defects; the two others, for some unknown reason, are quite highly abnormal on one side.

b) *S.B.*—We have seven feathers resulting from this experiment. In them all, the feathers tend to be normal except for a variable part of the apex involving one-ninth to one-quarter of the total length of the feather. We conclude that a lateral half of the dorsal surface of the papilla may produce a normal rhachis.

c) Three experiments were made to test the width of the rhachis field of the papilla: (*a*) In the first experiment a sagittal cut removed about two-thirds of the papilla on the right side, leaving one-third on the left. (*b*) In the second, a median strip of the papilla was removed, leaving two lateral strips. (*c*) In the third, lateral strips were removed, leaving a median strip about one-third the width of the papilla. Six operations of each kind were made.

The details of the results of these experiments are rather confusing, but the outstanding result is that a complete rhachis may develop from either a lateral, or the central, one-third of the dorsal surface. It thus becomes quite certain that the rhachis "field" occupies all the dorsal half of the papilla.

Summary.—From the various defect operations in the three planes there emerges very clearly the conclusion that the dorsal portion of the papilla, including both ectoderm and mesoderm, is necessary for the development of a normal rhachis and feather. Even a dorsal quarter may compensate for absence of the remainder of the papilla. On the other hand, a ventral half cannot compensate for absence of the dorsal half and does not develop a normal rhachis. The T experiments (Fig. 13, Pl. III) and the other operations involving only the apical half of the papilla (A type) clearly demonstrate an axial organization of the papilla also.

5. THE ISOLATION EXPERIMENTS

In these experiments the attempt was made to isolate parts of the papilla from one another by sagittal and frontal cuts along its entire length in the hope of producing two or more feathers in one follicle from a single papilla. Sagittal bisection yielded feathers from thirty-eight follicles, frontal bisection yielded feathers from twenty-five follicles, and division of the papilla into quadrants by both sagittal and frontal cuts yielded feathers from sixteen follicles.

In such experiments, as the parts remain in contact by wounded surfaces, there will be a tendency for them to heal together before actual regeneration begins. This was a very common condition. In a large number of cases only one of the isolates survived and developed; this was very clear in the case of frontal bisection because the dorsal and ventral isolates yield different results, as shown by the defect experiments; but in the case of sagittal isolates the distinction between complete healing-together and development of one half only is not easy to make.

In the case of frontal bisection the dorsal isolate usually suppresses the ventral one on account of the stronger growth of the former. Results do not differ from those of defect experiments. Similarly, the double bisection does not yield any results peculiar to itself. We therefore confine our attention to the results of sagittal bisection.

In this case, judging from the defect experiments, there is the possibility of producing two similar feathers in one follicle. There is no evidence of this in twenty-seven cases; but in the remaining eleven cases partial or complete twinning occurred. Two of these were complete twins from base to apex; in the remaining nine cases the feather pairs were more or less fused together from the base in varying degrees toward the apex, never in the reverse direction.

The specimen on the left in Plate VI (Fig. 24) is a photograph of one of the cases of complete twinning made in an early stage of development on the living bird. It will be observed that two feather cylinders, each with a separate sheath, emerge from a single follicle. These developed into separate feathers of unusual length (11 and 9.8 cm., respectively). The second case of complete twins show mirror imaging of brown areas in an otherwise black vane. In the other cases there was a single sheath for both components as in the twin pair on the right in Plate VI (Fig. 24). In such cases the twinning may be complete to the calamus (one specimen), to the fluff (one case), or in part of the vane only.

Figure 23 (Pl. VI) shows a case of twinning to the fluff region, which was plucked early for fear of losing it. The two rhachises approach each other and are apparently about to fuse in the common sheath. The vanes are not entirely normal in either partner but more nearly so in the right one. It will also be noticed that the external half-vanes of both partners are more nearly normal than the apposed vanes, owing presumably to interference of the latter with one another during development.

A very beautiful case of apical twinning is shown in Figure 22 (Pl. VI). The successive generations of this feather will be discussed later (p. 121).

6. RECOMBINATION EXPERIMENTS: CHIMAERAE

Feather papillae have the capacity for independent self-differentiation and can exhibit this capacity outside of their natural feather tracts. We have also seen that lateral halves of one sagittally divided papilla tend to reunite; the expectation of a union of lateral halves from different tracts, if the cut surfaces are apposed, is therefore not unreasonable. It should be possible, if technical difficulties can be overcome, to combine opposite lateral halves of papillae from different tracts on the same side of the body into a single papilla that will produce a feather with one half of the vane derived from one tract and possessing its characteristics, and the other half derived from the other tract and possessing its characteristics—in short, to produce a lateral feather chimaera.

Breast feathers and saddle feathers of the Brown Leghorn capon offer contrasting characters that suit them admirably for such an experiment (Pl. I, Fig. 6). The barbs of the breast feather bear barbules to their extreme tips, those of the saddle feather bear them only near their bases, and the remainder of the barb is devoid of barbules, giving the feather a "lacy" effect; the extent of the portion of the barbs bearing barbules in the saddle feather increases very gradually from a minimum apically until, near the region of the fluff, about a half of the length of the barb bears barbules. The color of the breast feather in the capon is blackish, that of the saddle feather golden brown. The fluffy region of the breast is relatively longer than in the saddle feather. The breast feather is broader and more rounded at the tip; the saddle feather narrower and acuminate.

The experiments were made by sagittal bisection of a breast papilla and removing one half as in the defect experiments, then transferring the opposite lateral half of a saddle papilla from the same side of the body and pressing its cut surface against the cut surface of the breast papilla in homologous orientation within the bottom of the opened breast follicle. The follicle repairs itself as usual.

Two experiments have been made. In the first experiment five saddle components were grafted to contralateral components of the breast tract. In the second, four reciprocal transplantations between breast and saddle tracts were made. Thus there were thirteen combinations in all. From these three typical lateral chimaerae were derived. Three breast follicles produced breast feathers, two saddle follicles produced saddle feathers, one saddle follicle produced both a breast and a saddle feather; three of the remaining four follicles produced feathers showing evidences of double composition, and the last feather developed only fluff.

One of the lateral chimaerae is shown in Plate I (Fig. 7). Its form is more like that of a saddle than that of a breast feather. The barbs on the left side are provided with barbules to their tips; and the pigmentation, though not so black as that of a normal breast feather, is darker and different in tone from that of the opposite side, which has the typical golden-brown color of the saddle. The vane on the right side is lacy, with axial distribution of barbules typical of a normal saddle feather. The fluff region is longer on the left than on the right side, corresponding to the normal proportions in breast and saddle feathers, respectively. At the apex there is not the least transition between breast-barb and saddle-barb, but typical ones are side by side. The second lateral chimaera differed mainly in the distribution of barbules, which was wider than usual on the saddle side; in the third the breast component is quite black.

Each component of the papilla in these cases has preserved its own tract specificity and field of action, but the whole constitutes a unity due to certain interchanges of effect between them. It would hardly be suspected, for instance, by observation of the chimaera alone that it was composed of half of a relatively short and broad feather and half of a relatively long and narrow feather (Pl. I, Fig. 6). There has been mutual accommodation of the components to produce a symmetrical result.

Danforth (1929 and 1937) described mosaic feathers near the margins of skin transplants between homologous feather tracts of different breeds of fowl. The effect was confined to color pattern and reappeared in successive generations. He rejects as inadequate the idea that "appropriate parts of two cut follicular anlagen were fortuitously brought into direct apposition" (Danforth, 1929) and suggests the possibility that nearby foreign tissue may in some way exercise a determining function. In his later paper (1937) this idea is further refined into the suggestion that migration of breed-different chromatophores may have been involved, which gains great probability from the later beautiful study of Willier and Rawles (1940) on the determination of feather pattern in the host by migration of the precursor cells of chromatophores from embryonic grafts. The technique employed in our experiments could well be adapted to further study of this problem.

7. SOME SPECIAL EXPERIMENTS

A. In sixteen cases in the breast tract the apical half of the papilla was reimplanted in its own follicle after complete removal of the basal half. Of eight cases in which a White Leghorn female was used, four did not regenerate; after 40 days two produced only a dwarfed group of vanelike barbs, and two produced portions of the vane 2.2 cm. and 2.7 cm. in length, respectively, which were not entirely normal. Of eight cases in which a Brown Leghorn male was used, six failed to regenerate; after 70 days one produced an approximately normal feather vane 4.5 cm. long, the other a half-vane feather 4 cm. long.

In five cases the apical halves of papillae of the breast tract were transplanted to follicles of fluff feathers dorsal to the breast tract from which their own papillae had been removed. Three failed to regenerate; after 52 days one produced a breast-type vane 3.2 cm. long that was approximately normal; the remaining one produced a short tuft of vanelike barbs without rhachis.

From these experiments it is obvious that the apical half of the papilla experiences considerable difficulty in establishing itself in viable condition, but, when it does so, its developmental capacity may be considerably more than its apparent prospective value judged from the defect experiments.

B. Dorsal halves of two papillae from the breast tract were applied together by their cut surfaces in the follicle belonging to one of them; thus in one partner the dorsoventral orientation is preserved; in the other, reversed. Out of five combinations one produced two approximately normal feathers 5.5 cm. long with ventral surfaces apposed, thus according to expectation. Three produced single normal feathers, and the fifth a dwarfed single feather with the rhachis forked about the middle of its length.

C. Ventral halves of two papillae from the breast tract were applied together by their cut surfaces in the follicle belonging to one of them. Out of five combinations, one produced two feathers of the half-vane type each 6.8 cm. long; another produced two feathers, of which one was of the half-vane type 8 cm. long and the other a typical tuft feather without rhachis 5 cm. long; and two produced single tuft feathers.

D. Kuhn (1932) produced a spiral twist of 180° in wing primaries of the pigeon by the following experiment: After plucking normal primaries, the long follicles dissected free nearly to the base were constricted by a thread tied tightly near the mouth of each, twisted several times, and bound by the free ends of the thread to neighboring primaries so as to preserve the twist. Although the follicles degenerated and were replaced by new ones, the feathers that resulted showed a half-spiral of rhachis and vanes with normally oriented base and the tip 180° reversed. Not being acquainted with the papilla, Kuhn attributed the result to distortion of the "matrix" from which the feather is derived. The condition persisted for three generations and gradually disappeared thereafter.

We have not succeeded in similar experiments on the Brown Leghorn but have produced similar spiral feathers in other experiments.

a) In a Brown Leghorn male the dorsal half was removed from three papillae of wing primaries (Experiment F.B.V.). In one case a half-vane feather resulted, repeated in the second generation now growing. This was an expected result. In the second feather, which attained a length of 16 cm. before plucking, the apex was twisted 180° at a mean distance of about 2 cm. from the tip. The second generation was similar, but the twist was somewhat reduced in the third generation now growing. The third feather was slightly twisted.

b) In a Brown Leghorn female three papillae of wing primaries were bisected sagittally. In one of these twin feathers developed. The second started as an apical twin, but one partner broke off; and the other, which attained a length of 15 cm., was twisted 180° at a mean distance of 4.5 cm. from the apex; the twisting was lost in the second generation. The third was a single feather rotated 180° at a mean distance of 5 cm. from the broken apex; the second generation, which attained a length of 19.5 cm. was likewise rotated 180°, but at a mean distance of only 3.5 cm. from the apex.

There are thus four cases of twisting out of six operations on wing primaries of the Brown Leghorn fowl in which the division of the papilla was longitudinal. Such cases have not been found in the much more numerous operations of similar nature on papillae of the breast tract. Although the papillae of the primaries are much larger than those of the breast tract, they are more slender in their proportions; they are also more difficult to get at. It may be that these conditions combine to give an unintentional twist to the papilla in the act of operating, but this is, of course, uncertain.

It is noteworthy that the operations were all on the left wing and that the direction of the spiral is the same in the four cases, which we designate as counterclockwise.

c) We attempted to produce spiraling in the breast tract by almost, but not completely, amputating the apical half of the papilla and rotating it 180° on the base and grafting it back. One out of eight feathers derived from this experiment showed a 180° spiral centered at the junction of the vane and the fluff, in addition to numerous elementary abnormalities of the barbs. The others exhibited the defects but not the spiral. The single case is, of course, not accidental. The twist disappeared in the second generation; the bird died after this.

Spiraling of the feather must be traced back to spiraling of the papilla if the results are to be interpreted in agreement with the other experiments. This interpretation has important theoretical consequences. It is noteworthy that the spiraling persists from only one to three generations and offers a contrast in this respect to other abnormalities (see Sec. 8 of this paper).

8. LATER GENERATIONS OF FEATHERS FROM OPERATED PAPILLAE

When a feather from an operated papilla is plucked for record, the papilla then proceeds to regenerate a new feather. These are likewise plucked and filed when mature, and so on for successive generations. The collection is, however, far from complete; when some of the birds ran at large in the summer the feathers were not plucked, and whole series were occasionally lost owing to death of a bird which might bear a considerable number of cases.

Among the defect experiments we have twenty-three cases in which both first and second generations have been preserved; in seven of these we have also the fourth generation. There are also seven additional cases in which first and third generations have been preserved. In these cases the general type of defect is repeated from the first to the fourth generations; defects are almost always more pronounced on one side, which remains the same from generation to generation. The details, but not the type, of the defects vary from generation to generation; but there seems to be no tendency toward a diminution of effect in four generations.

This result is so striking, and to some extent so unexpected, that individual cases deserve special attention. It might be expected that in the case of the T operation, which involves only amputation of the apical half of the papilla, the remainder would regenerate the missing part and that normal feathers would then be produced. However, the apex of later generations of such feathers remain defective. We have six cases in the second generation, after which the bird died, and two cases in the third generation from another bird. We have also five additional cases of F.A.D. operations in the second generation and two in the third generation in which the apical defects are repeated, and, finally, two cases of this experiment that produced limited half-vane type of defects in both the first and the second generations.

In the F.B.V. and S.B. experiments, which produce approximately normal feathers, those of the second generation are likewise approximately normal in the three cases recorded.

Seven half-vane feathers of the F.B.D. experiment were replaced by half-vane feathers both in the second and in the fourth generations (the third generation having been lost while the birds were running at large during the summer). In each case the half-vane was on the same side of the feather in successive generations. The photographs of first and second generations of one of these cases (Pl. V, Fig. 20) illustrate also the principle that the type of defect remains constant but details differ in successive generations; the half-vane is shorter in the second generation, and the free reversed barbs present in the first generation are lacking in the second generation.

The twins and chimaerae illustrate particularly well the persistence of composition of papillae established by operation. The four generations of the apical twin shown in Plate VI (Fig. 22) are strikingly alike in all major features. The rhachis is double from the fluff up, and it forks in two branches at approximately the same level in all. If the tips of the feathers appear different, this is due solely to differences in the length of the barbs in the apposed vane-halves in succeeding generations and to consequent variation in the degree of overlapping.

The chimaera illustrated in Plate I (Fig. 7) is of the second generation, which is practically identical with the first, and also with the third not yet plucked.

In general, the observations on the later generations demonstrate a surprising lack of capacity for regulation on the part of the papilla, except in the case of spiraling. It is possible that still later generations may demonstrate slow regulation, and the birds will be kept under observation as long as possible to determine the question.

9. THE OCCURRENCE OF ABNORMALITIES IN NATURAL FEATHERS

The abnormalities that we have produced by experiments have not hitherto been described as occurring in natural feathers. From this it may be concluded that they are at least very rare. We have examined over thirty-five hundred breast feathers from thirty-six Brown and White Leghorns and found that an average of 6.6 per cent of the feathers exhibited branched and bundled barbs. If branching at the extreme apex is eliminated for reasons stated below, the percentage in over two thousand feathers specially examined for this point was reduced to 1.7 per cent. The other principal elementary abnormalities of barbs were practically entirely absent.

Individual birds varied to an extreme degree in the percentage of feathers exhibiting these abnormalities. When the reasons for this variation were examined, we found that, out of a total of nine hundred and ninety-five feathers from seven birds not used in experiments and not previously plucked, only twelve showed occurrence of these abnormalities which were confined to the apex in ten cases; no abnormal feathers at all were found in three of these birds. In the remaining birds the percentage of abnormalities was greater; all the feathers examined were known to have regenerated within previously plucked areas; the birds had also been handled a great deal by strapping to the operation board during repeated examinations. Of about twenty-five hundred feathers from these birds a total of 9 per cent showed the abnormalities in question, which was reduced to 3.1 per cent when branching at the extreme apex was eliminated.

The general result of these examinations is to emphasize the extraordinary precision of the mechanism of development of feathers when naturally molted. After artificial plucking, the percentage of total abnormalities is multiplied at least seven times. The preponderance of apical abnormalities would seem to indicate that artificial plucking, which always involved some partly grown feathers, tends to produce slight injuries to the apex of the papilla. Even apart from this it would seem probable that the development of the feather may be slightly deranged by continual handling of the birds, which involves the danger of mechanical injury to the papilla.

10. SUMMARY OF RESULTS

1. The rhachis consists of a central, or medullary, component and two lateral, or ramic, components composed of expanded barb-petioles united serially by their fibrous extensions. The central rhachis itself is double in origin and to some extent in structure. It is prolonged apically a minute distance by the fused bases of the first two apical barbs.

2. Experiments were performed by operations on the papilla. Active papillae of regenerating feathers were used principally, but the resting papillae of completed feathers yielded similar results.

3. Transplantation experiments of various kinds prove that the papillae preserve an innate bilateral organization in whatever orientation or location they may be placed. Papillae of breast and saddle feathers carry their tract specificity when transplanted outside their own tracts.

4. Feathers with abnormal apexes develop from the basal half of the papilla after the apical half is removed by amputation. Other operations on the apical half of the papilla also produce apical defects of the feather.

5. Approximately normal feathers may develop from the dorsal half, the dorsal quarter, or a lateral half or even a third of the papilla, after removal of the remainder of the papilla in each case.

6. Tuft feathers with no rhachis, or half-vane feathers with a half-rhachis, are the typical products of development of the ventral half of papillae from which the dorsal half has been removed.

7. Similar twins may be produced by sagittal bisection of a papilla; all grades from apical duplication to complete duplication may result, according to the amount of healing after operation.

8. A single chimaera feather possessing characters of a breast feather in one half-vane and of a saddle feather in the other half-vane may be produced by fusing contralateral halves of a breast and a saddle papilla in one follicle.

9. Some of the above findings were confirmed by certain special experiments.

10. Operated papillae continue to produce the same types of abnormal feathers during successive generations of regenerated feathers.

V. THEORETICAL

1. INTRODUCTION

The mechanism of development of the feather is normally very precise, and the results in the development very constant. The experiments on the papilla derange the mechanism to a variable extent and with variable consequences. Previous deductions concerning the mechanism have been based mostly on two kinds of data: (1) on analysis of the normal development and of feather patterns and (2) on certain experiments dealing with the effects of hormones and conditions of nutrition and growth on the development and pattern of the feather. The present experiments differ inasmuch as the materials and conditions of development are kept as normal as possible and the mechanism itself is deranged.

The explanation of the abnormalities that we have described in the defect experiments is to be found in modifications of the normal development. The ideal way would be to follow the details of development of each type of abnormality; but we have not found it possible to do this. Even if this method were followed to its practical limit, the role of theoretical interpretation would not be greatly diminished; and it is possible, as a matter of fact, without in any way exceeding the limits of established biological principles, to make satisfactory correlations of the abnormalities with normal processes of development.

2. THE ELEMENTARY ABNORMALITIES

A. THE RHACHIS

Study of the normal development shows that the central rhachis and the barbs are of entirely different origin. The experiments also are conclusive concerning the distinction between central rhachis and barbs. Nevertheless, the strict correlation of abnormalities of the barbs with abnormality of the central rhachis in the experiments demonstrates that the latter is the principal factor that controls the tangential movements and the normal behavior of the barbs in development. The interpretation of the elementary abnormalities of the barbs is, therefore, to be sought mainly in the abnormalities of the rhachis produced by the operations. In natural feathers, however, elementary abnormalities of barbs of a relatively simple kind may occur in the presence of a normal rhachis (p. 122); they appear to be associated with localized disturbances of tangential movements independent of the rhachis, judging by correlated modifications of spacing of the barbs concerned.

In our classification we have reduced abnormalities of the rhachis to two main types, viz., absence of both right and left sides and absence of one lateral half; both are graded as to extent in an apicobasal direction. On a priori grounds other types of abnormalities would be possible, such as isolated lateral (ramic) components or multiple rhachises. But it is very doubtful that these occur, though it is true that repeated branching, union, and bundling of barbs may simulate multiple conditions of the rhachis (cf., e.g., Pl. VII, Fig. 27).

Bilateral absence of the rhachis in any degree is readily diagnosed by the tufted arrangement of barbs above the level where the rhachis terminates and by the regular bilateral arrangement of barbs proximal to this point (Pl. VIII, Fig. 28, and Pl. IV, Fig. 19). Diagnosis of a half-rhachis rests on the regular arrangement of barbs to form a half-vane on one side and the reversed and tufted arrangement of barbs on the opposite side. Two independent half-rhachises do not form separately in our experience. Incompleteness of fusion of lateral halves does occur, however.

None of the various forms of branching, union, or bundling of barbs produces a structure that has more than superficial resemblance to the central rhachis, which has the unmistakable properties of attracting barbs to itself, of stimulating the formation of barb petioles, and of independent basal growth and enlargement. The experiments thus demonstrate that the central rhachis is an independent segregate with properties peculiar to itself.

B. THE BARBS

Each barb is an independently growing and differentiating unit. Cell division is confined to a very short segment at its base; above this zone keratinization sets in very rapidly and fixes conditions so that branching, union, bundling, and inversion of barbs becomes impossible. The modifications of normal development that lead to abnormalities are located in the zone of cell division. But this zone itself must be further subdivided; in its more distal section the segregation of cells for stem of the barb, for barbules, and other purposes is accomplished, and their further multiplication occurs. Proximal to this it is necessary to postulate in each barb a center of undifferentiated cells in juxtaposition with the collar from which all the various cells of the barb are derived. This we shall call the *organization center of the barb.*

The individual barbs concerned in the abnormalities are complete as to organization, which could not happen if the abnormalities arose after cell differentiation had occurred. We conclude, therefore, that the elementary abnormalities owe their origin to behavior of the organization centers of the barbs.

In normal development the bases of the barbs are set on the collar as closely together as is consistent with their mutual independence (cf. numerous photographs in the paper by Lillie and Juhn, 1938). It is one of the theses of the present paper that the normal regular spacing of the barbs is interfered with either by defect or by excess under the conditions produced by the experiments.

"Branching" will occur if two adjacent organization centers fuse and then continue to act as one; the two original barbs will be carried away from the collar by the axial growth of the whole cylinder and will be attached as branches to a single barb derived from the fused organization center. Repetition of this process involving the bases of such branched barbs and others will lead to complex branching systems (Pl. III, Fig. 14, and Pl. VIII, Fig. 29). The form of the branching will vary according as combinations of single, double, triple, or more complex centers are concerned.

"Union" will occur between barbs if the organization center of a single barb divides and the two centers then grow independently. Repeated division may then produce systems of considerable complexity (Pl. VII, Fig. 27). Union is very common as a sequence to branching, i.e., to previous fusion of organization centers, thus producing bundling. The bases of reversed barbs also are usually divided repeatedly. Union is thus common in association with other abnormalities, but it is also found separately, though rarely (cf. Pl. VII, Fig. 26).

"Bundling" will occur if fusion of two centers is followed by division of the conjoined center. The time interval between fusion and division will determine the length of the common section between the branches and the united barbs. In the case illustrated in Figure 26 (Pl. VII), three apexes of barbs are concerned and only two bases, from which it would follow that the number of organization centers entering into a fusion does not determine the number of subsequent divisions of the fusion center.[8] Fusion appears usually to produce a center of independent value. This conclusion is supported by a large number of complex cases. The repetition of bundling along an isochrone, as shown in Figure 29 (Pl. VIII) and observed in other cases, is suggestive.

The causes of three of the four major barb abnormalities are reducible to two simple biological principles of very wide occurrence, viz., the production of a single undifferentiated primordium by fusion of two, and the production of two primordia by division of a single undifferentiated primordium. What are the underlying conditions created by the operations that induce these unusual phenomena?

They are associated, as we have seen, with absence either of both halves of the rhachis or of one lateral half only and with the extent of these defects, which are graded in different cases from its apex toward its base. It is always the apex of the rhachis that is first affected; there is consequently delay in tangential movements of barbs until the rhachis forms. During the delay barbs grow vertically side by side. If the collar should retain its primitive capacity to form new barb apexes at any locus, and new ones continue to arise, irregularities both in order of origin and in spacing of barbs might be expected, owing to the altered conditions. Abnormalities may be referred to such irregularities.

Such a general explanation would remain purely formal if we were not able to deduce from the structure of the feather the order of origin of its barbs. When a vane or half-vane is present, the order of origin is the same as the order of succession on the rhachis. When the rhachis is absent and the barbs grow vertically in consequence, the relative lengths of the barbs is the clue to the order of their origin according to the principle of equal rates of axial growth around the cylinder.

We may test this out on Figure 17 (Pl. III), for example. On the defective side we have a complex of branched and bundled barbs attached by a single base to reversed barbs. The complex is much longer than the reversed barbs and, therefore, of correspondingly earlier origin. But the reversed barbs took their origin between the complex and the rhachis, that is, in a more dorsal position on the collar, which is the exact opposite of the normal order of origin. Tuft feathers and the defective side of half-vane feathers furnish additional examples.

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[8] By contrast, in natural feathers, where we have examined numerous cases of bundling (which have never involved more than four barbs), the number of the apexes concerned always agrees with the number of bases. Moreover, the arrangement of the apexes is always repeated in the bases but as a mirrored image, i.e., in inverse order. The reason for this difference is obscure.

There can be no doubt that regular succession of barbs on the collar as in the development of the normal feather is not found in absence of the rhachis. We are not prepared to say that there is no regularity but only that whatever degree of regularity there may be is subject to many exceptions.

The absence of localization of a restricted region of origin of new barb apexes, comparable to the ventral triangle in normal development, and cessation or irregularity of tangential movements, are not in themselves an explanation of the unusual behavior of the organization centers in fusing and dividing. But as they lead to crowding of the organization centers on the collar, or the reverse, they may become significant. Much-branched barbs usually have a very attenuated common base, as in Figures 14 (Pl. III) and 29 (Pl. VIII), for instance, which suggests a preceding condition of crowding of barb bases favorable to fusion. In the case of union of barb bases we do not observe such an attenuation of the bases, and this suggests the opposite condition of ample space for the bases precedent to division of the organization center.

C. THE THEORY OF REVERSED BARBS[9]

Reversed barbs occur usually in connection with a half-rhachis; they are attached opposite to the half-vane and belong in a series of tufted barbs (Pl. V). This situation at once furnishes the clue to their interpretation. We have shown that in half-vane feathers the order of origin of barbs is irregular on the defective side and that they may arise in any part of the collar. Thus they may arise at any time adjacent to the half-rhachis. If we made the assumption that the influence of the half-rhachis in attracting barbs to itself, which results in the formation of the regular half-vane, extends also in a slight degree to the defective side, a barb arising from a center close to the half-rhachis would fuse with it by its newly formed apex. This barb would then act as a block to fusion of any more remote ones on account of the rapid progress of keratinization. We shall call it the "first reversed barb" (Fig. 5, 1).

On the same assumption late-formed portions of the same barb would likewise fuse with the half-rhachis, involving thus its entire length (Fig. 5, 1), but it would not constitute an independent reversed barb. In such a case the only evidence of the first reversed barb would be a series of barbules along the defective side of the half-rhachis. Barbules are, in fact, usually found along the defective side of a half-rhachis, and there is no other source for them than from the defective side.

The first independent reversed barb (Fig. 5, 2) arises by division of the organization center of the first reversed barb, on the same principle as the origin of united barbs, thus establishing a center independent of the rhachis. According to its mode of origin, and as an observed fact, its point of attachment to the half-rhachis succeeds a series of barbules on the half-rhachis, which represent the apex of the first reversed barb; in rare cases this may reveal its barb nature by a minute free tip. Either center may divide again; in the

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[9] The theory presented here is the result of much cogitation and discussion between the authors. We believe that it is consistent with all the facts and with the theory of united barbs. An alternative theory, presupposing separate origin of each barb of a series of reversed barbs, would have to postulate the successive origin of new centers between the first reversed barbs and the half-rhachis, which is impossible as long as the first reversed barb continues to grow along the half-rhachis.

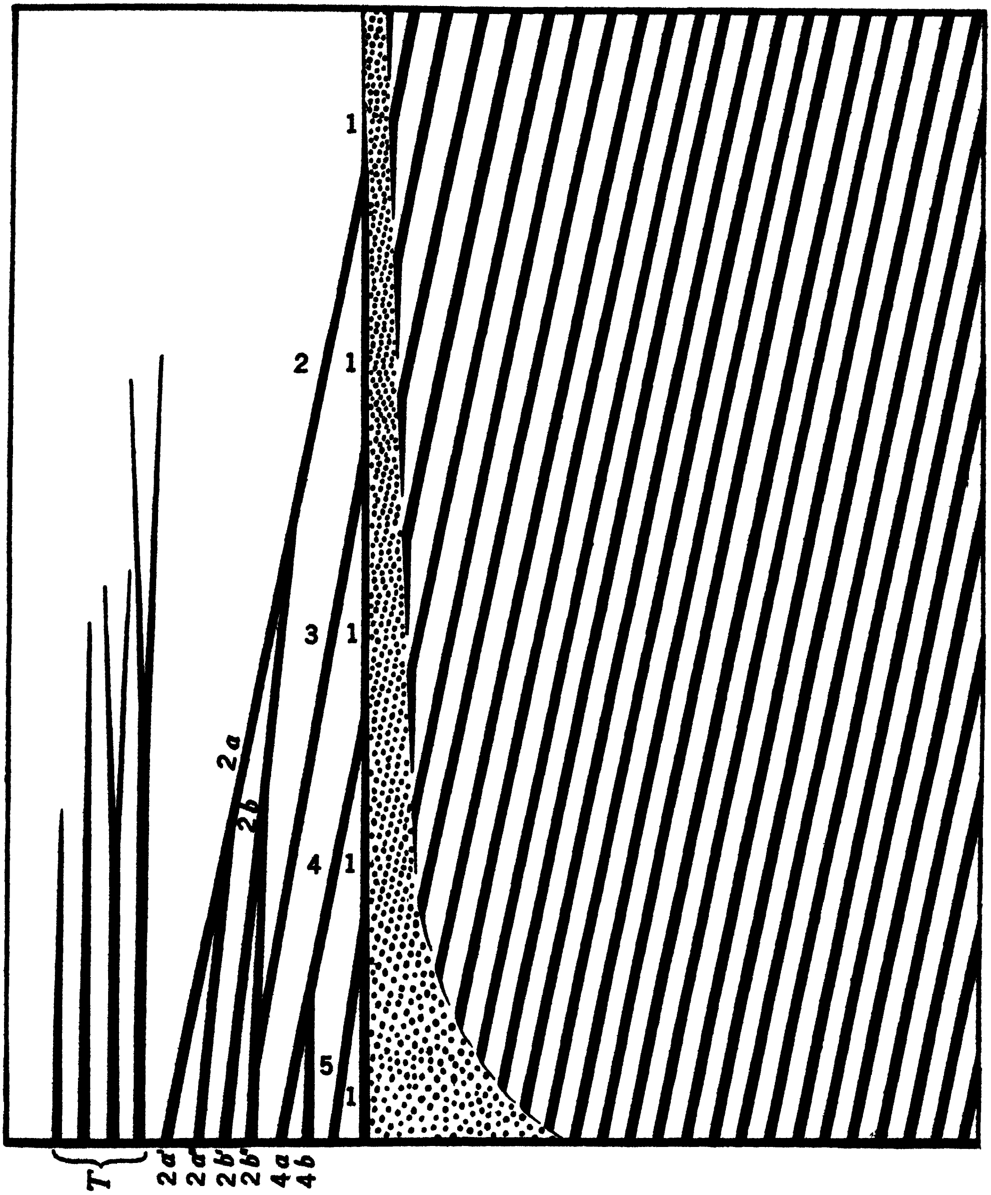

Fig. 5.—Diagram to illustrate the theory of the origin of "reversed" barbs in half-vane feathers, as stated in the text; it represents an advanced stage in the development of a series of reversed barbs, the time order of which is to be read from above downward. The line across the bottom of the diagram represents the position of the margin of the collar. The right side of the diagram is based on a total preparation of a normal feather cylinder split along one side and mounted flat with the barb ridges up. The number of barbs on this side has been reduced, and the barbules are omitted. Barb petioles are represented incompletely. The stippling represents the right lateral half of the central rhachis, which is absent on the left side. The left side of the diagram is a purely theoretical reconstruction based on half-vane feathers. To the left of the reversed barbs four "tuft barbs" are represented, two of them branched (cf. description in the text).

case of the independent barb two united barbs arise (Fig. 5, *2a* and *2b*), and in the case of the first reversed barb a second independent (Fig. 5, 3) and a fused barb. This process may be further repeated (Fig. 5, *2a′*, *2a″*, etc.). All such barbs are united apically to the half-rhachis.

This account corresponds well with the specimen shown in Figure 21 (Pl. V). But other complications may arise. Two centers of independent reversed barbs may secondarily fuse (Fig. 5, 3 with *2b″*), thus producing branching as in Figure 17 (Pl. III); or the center of the most lateral reversed barb may fuse with an adjacent single or branched barb, as also shown in the same figure. But however complex the results, the principle of origin of reversed barbs remains the same.

The most apical of the independent reversed barbs may acquire free bases in the course of development (Pl. V, Figs. 20 and 21) and stand out stiffly from the half-rhachis. It is obvious that such free barbs have ceased to grow at a rate sufficient to keep up with the axial elongation of the feather as a whole and are thus pulled loose from the collar. Most of the reversed barbs, however, maintain their connection with the collar, and thus their bases remain within the feather cylinder. A third condition (looped) is found in feathers in which the rhachis becomes bilateral at a certain distance from the apex. In this condition the base is attached on the same side of the rhachis as the apex, owing to the resumption of tangential movements on its own side (Pl. III, Fig. 17).

There is also a curious reversal of sidedness characteristic of the barbules of reversed barbs. In the vane of the normal feather the distal barbules bear hooklets, but the proximal barbules do not (Pl. II, Fig. 10). The two series of barbules differ also in other respects. In the developing barbs of the feather cylinder the side next to the rhachis normally develops the distal type of barbule. But in reversed barbs it is the side away from the half-rhachis that bears barbules predominantly of the distal type, though they may occur sporadically on the side next to the half-rhachis. This has the remarkable consequence that in half-vane feathers, whereas barbules of the proximal type occur on the barb petioles of the dominant side, on the opposite side of the same half-rhachis the barbules are predominantly of the distal type.

It is exactly as though the differential forces, which may be referred to the rhachis, that determine the sidedness of the barbules with reference to the barb, extend their influence in the case of the half-rhachis on to the defective side. There is, in any case, a reversal of the direction of action of the differential forces on the defective side.

The presumed differential action of the rhachis in determining the sidedness of barbules receives a test in the case of tuft feathers, i.e., in absence of the rhachis. The isolated barbs of such feathers carry two series of barbules above the fluff which are widely spaced, smaller than normal, and usually not differentiated. If some of them bear hooklets, they are placed indifferently on either side. But occasionally small groups of interlocking barbs occur in tuft feathers; in such cases one side of each barb bears barbules similar to the distal type of normal barbs, and the other barbules of the proximal type, and the "sidedness" is the same in all members of the interlocking group, though there is no basis of reference to denominate the rows distal and proximal. It would seem to follow that determination of distal and proximal types of barbules, though usually controlled by differential action of the rhachis, may occur without such action.

3. THE THEORY OF TANGENTIAL MOVEMENTS

Lillie and Juhn (1932) distinguished two kinds of apparent movements of barbs during their growth. Both have reference to the collar. The first is due to growth in length of the barbs, by which the distance from the apex of the barb to the collar is constantly increasing. The second is the apparent migration of the growing base of the barb along the collar from its ventral point of origin to the rhachis situated dorsally. Fraps and Juhn (1936) subsequently denominated these movements as "axial" and "tangential," respectively. The axial growth of the cylinder as a whole involves all its parts at equal rates (Lillie, 1940); hence it remains straight, and the axial growth of barbs is equal to that of the cylinder, except for the slight differential introduced by their tangential movements, in which the cylinder as a whole does not share. The position of the developing barb in the wall of the cylinder becomes a half-spiral owing to the combination of axial and tangential movements of the barbs (Lillie and Juhn, 1938, Fig. 3, diagram).

Hosker (1936) and 'Espinasse (1939) objected to the conception of tangential movements and pointed out that the conception of movement of the bases of the barbs along the collar becomes unnecessary if it is assumed that the barb-forming ridges are cut out of the collar by grooves that have a dorsal inclination; the prolongation of these grooves, as axial growth proceeds, would be progressively more dorsal in position until they met the rhachis. 'Espinasse calls this the classical theory and attributes it to Davies (1889) and Strong (1902), though on what grounds it is difficult to see. He does not suggest any extrinsic differentials determining the spiral direction of barb growth or any explanation of important exceptions, which we had previously described.

There is no question as to the translocation of the base of each barb from its point of origin, which is on one or the other side of the ventral triangle, except in the case of apical barbs, to the rhachis. There is, however, much difference of opinion as to the factors which produce translocation. Lillie and Juhn (1932) regarded it as part of a process of total concrescence of the halves of the collar in the mid-dorsal line involving the primordium of the rhachis regarded as coextensive with the barbs. A growth center for each barb was accordingly postulated in the collar. Fraps and Juhn (1936) brought cogent arguments against the idea that the primordium of the rhachis is coextensive with the complement of barbs in the collar. Lillie and Juhn (1938) accepted their idea that the primordium of the rhachis has a short, spreading base in the collar entirely independent of the barbs.

This left the theory of tangential movement of the barbs without any basis of support. Fraps and Juhn accounted for it by assuming that the dividing cells concerned are "characterized by a vectorial displacement" (1936, p. 371), without reference being made to any extrinsic differentials. They also dismissed the idea of individual growth centers of barbs as unlikely. Lillie and Juhn (1938), without any reference to vectorial displacement of dividing cells, emphasized the relation between the time of origin of the rhachis and the beginning of tangential movements and the necessity for correlation between axial growth of the shaft and the completion of tangential movements of the barbs; they also suggested a possible function of physiological gradients in the collar and the *vis a tergo* of the successive origin of new barbs at the ventral triangle as another possible contributing factor. They replaced their original theory of growth centers of barbs in the collar by the conception that the bases of formed segments of barbs act as organizers of ramogenous material arising from the collar.

It is now clear from the present experiments that the principal differential determining tangential motion of the barbs resides in the rhachis; for in absence of the rhachis tangential movements are entirely absent. Each half of the rhachis acts independently as shown by the half-vane feathers. However, the occurrence of the principal elementary abnormalities of the barbs in natural feathers when the rhachis appears entirely normal indicates clearly that slight disturbances in the regularity of tangential movements may occur independently of the rhachis in restricted loci.

The absence of tangential movements has far-reaching consequences; the barbs grow in a strictly axial orientation with reference to the cylinder; the number of barbs is greatly reduced in comparison with the normal; each barb may grow indefinitely in contrast to the normal termination of growth by attachment to the rhachis and formation of a petiole; new barbs may form apparently at any unoccupied locus in the collar, not merely in a restricted ventral locus as in the normal; division and fusion of barb centers, which are rare in normal development, become usual occurrences.

4. THE ORGANIZATION OF THE PAPILLA

The experiments demonstrate conclusively that the papilla controls the orientation and general development of the feather either directly or indirectly. However, it remains unaltered morphologically throughout the period of development of any feather and is ready to begin the regeneration of a new feather all over again whenever a feather in any stage of regeneration is plucked. This presents a problem in morphogenesis somewhat out of the usual order; though it has analogies to other cases of regeneration, there is a real difference in the discreteness of the primordium.

We shall begin by inquiring whether the control exercised by the papilla resides in its massive mesodermal component or in its delicate epidermal covering (Pl. III, Figs. 11 and 12). When we consider the fact that all the operations leave a large wounded surface which requires to be covered by proliferation and extension of the ectodermal cells and that this occurs in all the planes of the operations, it is impossible to believe that the proved prospective values that pertain to isolated portions of the papilla can proceed directly from such a flowing substratum as the epidermal covering. The repetition of defects from generation to generation of feathers from operated papillae likewise requires a more substantial basis of support. For these general reasons we proceed on the assumption that the morphogenetic control exercised by the papilla resides in the mesoderm.

It expresses itself, however, exclusively through the Malpighian layer of epidermal cells that cover its surface. These proliferate rapidly, and the external layers soon form a keratinized sheath over the germ from apex to base. Beneath this by continued multiplication of the Malpighian cells, especially toward the base, the collar becomes evident, and the barb apexes form.

The ectoderm covering the surface of the papilla is totipotent as a whole with reference to the feather, and we believe that this pertains to all its parts. Therefore, the prospective significance of these cells depends on induction by the mesoderm.

The question then becomes one concerning the time and extent of induction by the mesodermal papilla. The fact that the papilla continues to produce the pulp filling the interior of the feather cylinder, on which the nutrition of the developing feather depends, shows that the mesodermal papilla is necessary throughout development, but it does not show that the morphogenetic functions of the papilla are likewise continuous. Indeed, an experiment of Woitkewitsch demonstrates conclusively that this is not the case.

In Woitkewitsch's experiments (1936) regenerating wing coverts of pigeons of 10–13 days were pulled out as far as the mouth of the follicle and then replaced either in normal orientation or rotated 180°. Over half of eighty-three feathers, which were replaced in normal orientation, resumed growth, always in normal orientation. In one hundred and fifteen operations, in which the feathers were rotated 180° before replacement, thirty-three resumed growth and retained the reversed dorsoventral orientation. This interesting experiment shows that reversal of the collar produces reversal of subsequently developing parts of the feather, even though the papilla retains its normal orientation.

Presumably this principle goes back to the beginning of the dorsoventral organization of the collar. The first evidence of this is the arrangement of the barbs in the cylinder, which are heavy in the dorsal half and deeply pigmented in the Brown Leghorn, making a clear distinction between dorsal and ventral halves of the cylinder. The position of the rhachis is determined within this previously existing dorsal field. We are unable at present to find any earlier morphological indicators of bilateral symmetry in the ectoderm; but the induction of the dorsal field must be earlier.

We conclude that with the determination of the dorsal field the ectoderm becomes a self-differentiating system, within which secondary systems governing the mechanism of development of the feather arise. This conclusion would limit the morphogenetic functions of the papilla to induction of a bilaterally symmetrical dorsal field in the ectoderm, including, of course, the antecedent processes of multiplication of cells and of growth in the ectoderm. How is this to be reconciled with the localization of abnormalities produced in the defect experiments?

The induction of a dorsal field in the ectoderm presupposes a corresponding organization field, or organizer, in the mesoderm of the papilla. The extent of this field is clearly indicated in the experiments. The removal of the apical half of the papilla results in abnormalities in the apex of the feather; therefore, the organizer extends to the apex of the papilla. The fact that the apical half of the papilla transplanted to an empty follicle produces a small symmetrical vane which may be of considerably greater extent than the defects produced in its absence is evidence against the idea of precise localization of the axis of the feather in the papilla.

By sagittal bisection of the papilla all grades of twinning may be produced, and it is only in those cases in which the halves remain separate from the apex through the base of the papilla that completely separate twins are produced. It follows, therefore, that the organizer extends also to the base of the papilla.

We have shown that a lateral third of the papilla may induce a complete dorsal field in the ectoderm, which gives some idea of the lateral extent of the organizer; and the fact that half-vane feathers develop so frequently from ventral halves indicates that the lateral margins of the organizer must extend to the ventral half, for some portion of the organizer is necessary to explain induction of the half-rhachis.

If the induction field thus includes the dorsal half of the papilla, should we ascribe to it a mosaic organization of its own to account for localization of effects? This interpretation would produce endless difficulties if it were assumed that the elements of such a mosaic act in a time sequence parallel to the sequence of development in the feather, and it is, indeed, excluded by the results of Woitkewitsch's experiment.

We cannot be dealing with a real mosaic structure of the organizer, but at most with a field possessing gradient properties that induces a field with gradients in the ectoderm. To relate such a conception to the appearance of abnormalities having definite localization and sequence in time introduces difficulties of its own. In considering these we emphasize the fact that, as shown by the tuft feathers, the induction of a dorsal field in the ectoderm is not necessary for formation of barbs.

In the case of amputation of the apical half of the papilla we have seen that the time of emergence is at least 18 days in the case of the breast feather instead of 7 to 9 days as in the normal. If the induction of the dorsal field in the ectoderm has undergone a corresponding delay, a certain number of barbs would have formed without benefit of this field and be subject to the development of elementary abnormalities. When induction of the dorsal field in the ectoderm becomes effective and the rhachis arises, the remainder of the development of the feather will become normal, and the abnormal barbs will be carried as a defective apex. Thus the apical location of defects in this experiment does not require the assumption of corresponding localization in the organizer but can be explained by delay in the inductive action of the organizer, the duration of which determines the extent of the apical defects.

The localization problem involved in the lateral defects of half-vane feathers may also be explained on a quantitative basis: a lateral half, or even a third, of the organizer may induce a complete dorsal field in the ectoderm. But when the attempt is made to remove the dorsal half of the papilla, and hence the entire organizer, a smaller lateral margin of the organizer may be left behind in the ventral half from which half-vane feathers are derived. This lacks the power to control the organization of the entire ectoderm, and hence organized and unorganized portions lie side by side. It is a significant fact that we have in our records no case of a diminished bilateral rhachis forming a small symmetrical vane with adjoining tufted barbs, whereas we have twenty-one cases of the half-vane type. We are forced, therefore, to the conclusion that lateral margins of the dorsal organizer of the papilla induce lateral vanes only; and from the fact that these may be either right or left (ten right and eleven left in our cases) we also conclude that the right and left margins induce right and left vanes, respectively.

As material for study of morphogenesis, feathers possess the unusual, and indeed unique, advantage that the operated germ (papilla) functions in the production of successive generations. The remarkable fact is that the same defects continue to occur as in the first generation. It is difficult to reconcile the continued lack of power of regulation of the papilla thus expressed with the quantitative interpretation of localization in the dorsal organizer that we have given. Nevertheless, we believe that a quantitative theory is the best guide for further research.

POSTSCRIPT

Since the present paper was completely printed, our attention was called to T. H. Morgan's paper (1917, Inheritance of number of feathers of the fantail pigeon, Amer. Nat., 52:5–27), in which "split" tail feathers are described and illustrated in F_2 and back crosses between fantail and homer pigeons. Such feathers present all degrees of doubling such as we have described in our sagittal bisections of the papilla (p. 117). Morgan's general conclusion is that "we are dealing with a single rudiment that has split at a very early stage into two parts that have completed themselves as whole feathers, so far as this intimate union in the middle line of the bud permitted. There are no indications that the split feather is due to the union of two separate rudiments that have been pressed together so closely as to interfere with the full development of each when they came in contact."

The comparison furnishes a good example of a phenotypic result that may be attained in identical form by the operation of genetic factors or of somatic operations. It is interesting to note that in twenty out of twenty-four of Morgan's cases "the doubling occurs near the middle of the tail," for this suggests that a study of fantail embryos may furnish an ontogenetic explanation of the fantail condition.

LITERATURE CITED

DANFORTH, C. H. 1929. The effect of foreign skin on feather pattern in the common fowl (*Gallus domesticus*). Roux Arch. f. Entw'mech., **115**:242–52.

———. 1937. Pigment cells in heterogeneous feathers. Anat. Rec., **68**:461–68.

DAVIES, H. R. 1889. Die Entwicklung der Feder und ihre Beziehungen zu anderen Integumentgebilden. Gegenbaurs morph. Jahrb. **15**:560–649.

'ESPINASSE, P. G. 1939. The developmental anatomy of the Brown Leghorn breast feather, and its reactions to oestrone. Proc. Zoöl. Soc. London, A, Part IV, **109**:247–88 (published January 24, 1940).

FRAPS, R., and JUHN, M. 1936. Developmental analysis in plumage. II. Plumage configurations and the mechanism of feather development. Physiol. Zoöl., **9**:319–77.

HOSKER, A. 1936. Studies on the epidermal structures of birds. Phil. Trans. Roy. Soc. London, B, **226**: 143–88.

JUHN, M., and FRAPS, R. 1936. Developmental analysis in plumage. I. The individual feather: methods. Physiol. Zoöl., **9**:293–317.

KUHN, O. 1932. Entwicklungsphysiologische Untersuchungen an der Vogelfeder. Roux Arch. f. Entw'-mech., **127**:456–541.

LILLIE, F. R. 1940. Physiology of development of the feather. III. Growth of the mesodermal constituents and blood circulation in the pulp. Physiol. Zoöl., **13**:143–75.

LILLIE, F. R., and JUHN, M. 1932. The physiology of development of feathers. I. Growth-rate and pattern in the individual feather. Physiol. Zoöl., **5**:124–84.

———. 1938. Physiology of development of the feather. II. General principles of development with special reference to the after-feather. *Ibid.*, **11**:434–48.

STRONG, R. M. 1902. A case of abnormal plumage. Biol. Bull., **3**:289–94.

WILLIER, B. H., and RAWLES, M. E. 1940. The control of feather color pattern by melanophores grafted from one embryo to another of a different breed of fowl. Physiol. Zoöl., **13**:177–99.

WOITKEWITSCH, A. A. 1936. Über den Ursprung des Materials bei der Bildung der Federanlagen. Roux Arch. f. Entw'mech., **135**:664–70.

PLATE I

FIG. 6.—Normal breast (*A*) and saddle (*B*) feathers of Brown Leghorn capon; dorsal view. ×1⅒.

FIG. 7.—Second generation chimaera produced by grafting a lateral half of a papilla of the saddle tract to the contralateral half of a breast papilla in a breast follicle on the same side of the body, Brown Leghorn capon. The left side of the chimaera is from the breast, and the right side from the saddle (see p. 118); age of feather, 94 days; dorsal view. ×1⅒.

PLATE II

FIG. 8.—Completely formed breast feather of a Brown Leghorn capon dissected after KOH treatment, showing lateral, or ramic, components of the rhachis stripped off from the central component with the whole complement of barbs *in situ;* dorsal view. Natural size.

FIG. 9.—Enlarged portion of left half-vane of a breast feather of a Brown Leghorn capon separated from the central rhachis, including parts of nine adjacent barbs forming the ramic component of the rhachis; the petioles of the two lower barbs are partly detached; cleared preparation in balsam; ventral view. ×20.

FIG. 10.—Completely detached barb from the left half-vane of a dissected breast feather, Brown Leghorn capon; cleared preparation in balsam; ventral view. ×40. *p.*=petiole; *d.r.*= distal row of barbules; *p.r.*=proximal row of barbules; *h.*=hooklets; *f.*=fibrous extension of the petiole; the arrow indicates the termination of the medulla of the barb.

PLATE III

FIG. 11.—Longitudinal section of an active papilla of a breast feather fixed immediately after plucking, Brown Leghorn capon; 25 days' regeneration. ×50.

FIG. 12.—Longitudinal section of a resting (inactive) papilla of a breast feather fixed immediately after plucking, Brown Leghorn capon. ×50.

FIG. 13.—Whole view of a breast feather from Experiment T in which the entire apical portion of a papilla was removed, Brown Leghorn hen; dorsal view; age of feather, 66 days post operation. ×⅞.

FIG. 14.—Enlarged apex of feather shown in Figure 13 showing apical defects in the form of missing barbs (right side) and branched barbs (both sides); left side dominant; dorsal view. ×3¾.

FIG. 15.—Enlarged apex of feather from Experiment T, White Leghorn hen, breast. Its apical defects consist of a short looped barb and branched barbs. Dorsal view; age of feather, 64 days post operation; length of entire feather, 8.5 cm. ×3¾.

FIG. 16.—Enlarged apex of feather from Experiment F.A.D. in which the dorsal half of the apical portion of a papilla was removed. White Leghorn cock, breast. Its apical defects consist of missing and branched barbs and a "ladder" due to repeated union of barbs. Left side dominant; dorsal view; age of feather, 66 days post operation; length of entire feather, 8.5 cm. ×3¾.

FIG. 17.—Enlarged apex of feather from Experiment F.A.D., Brown Leghorn hen, breast. Apical defects consist of reversed, branched, and looped barbs on the right. Note the group of branched barbs with bundling and the group consisting of one branched and one single barb attached to the reversed barbs. Dorsal view; age of feather, 51 days post operation; length of entire feather, 7.5 cm. ×1½.

PLATE IV

FIG. 18.—Tuft feather from an experiment in which a large part of the basal portion of a papilla was removed, Brown Leghorn capon, breast; age of feather, 82 days post operation. Natural size.

FIG. 19.—Tuft feather completely devoid of a rhachis; from F.B.D. operation on a resting breast papilla, Brown Leghorn male; age of feather, 73 days post operation. Natural size.

PLATE I

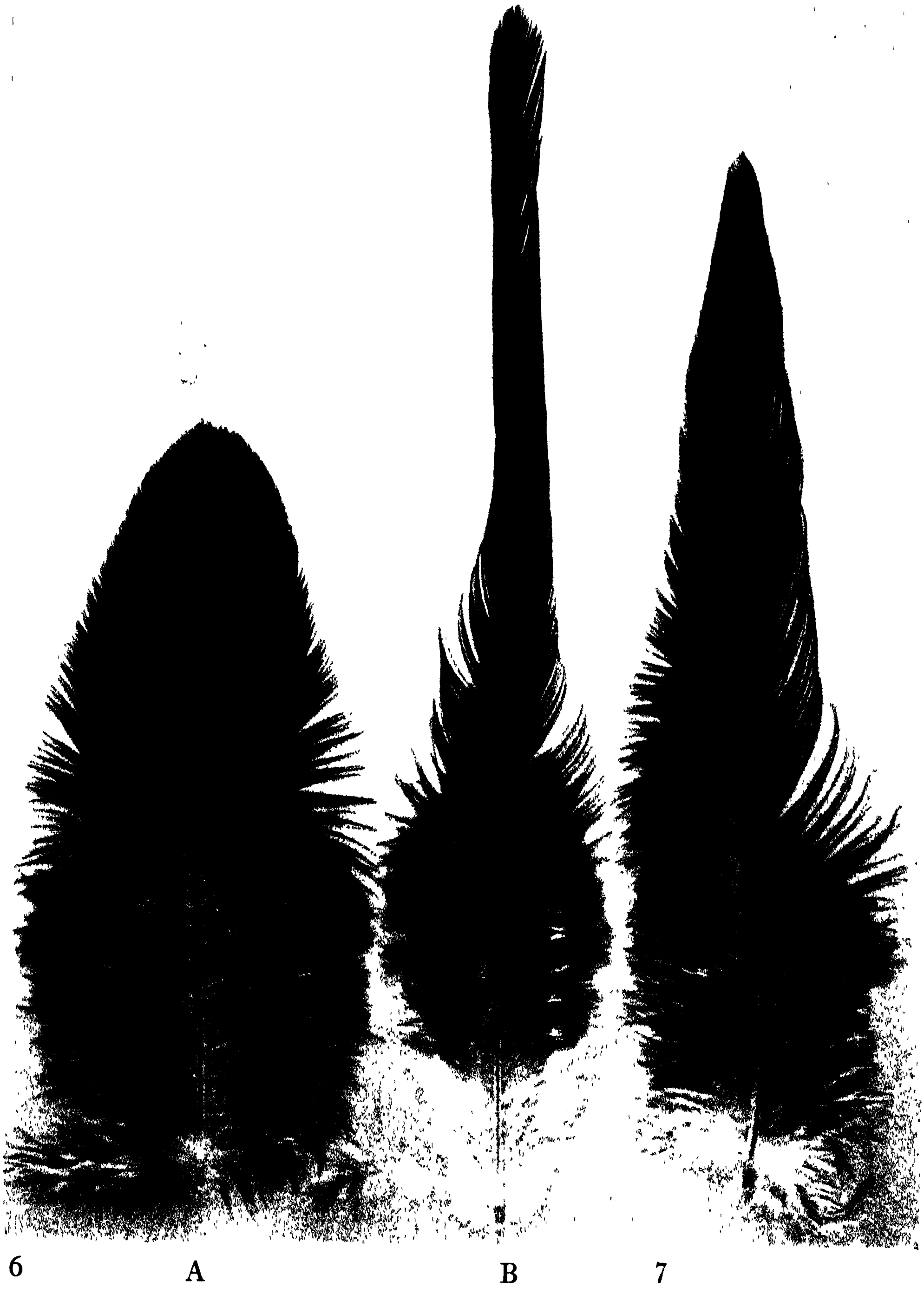

PLATE II

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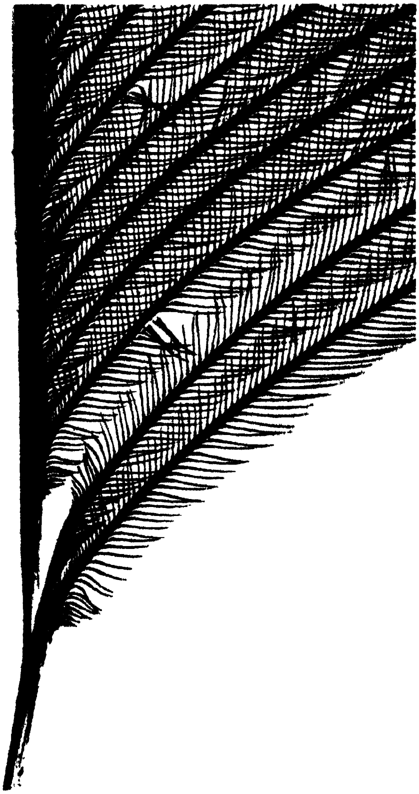

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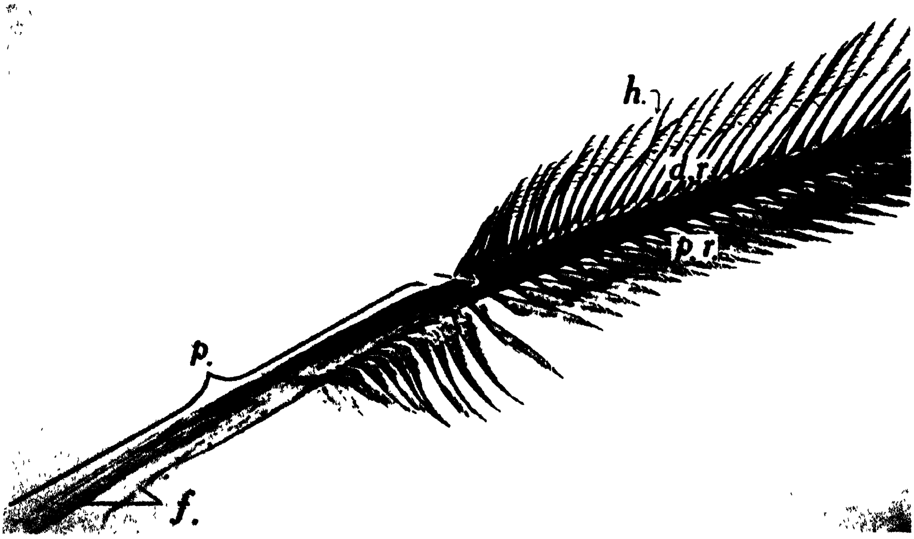

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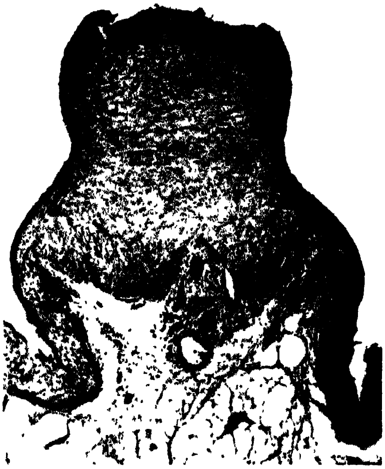
PLATE III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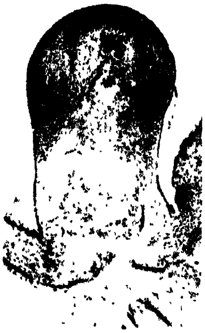
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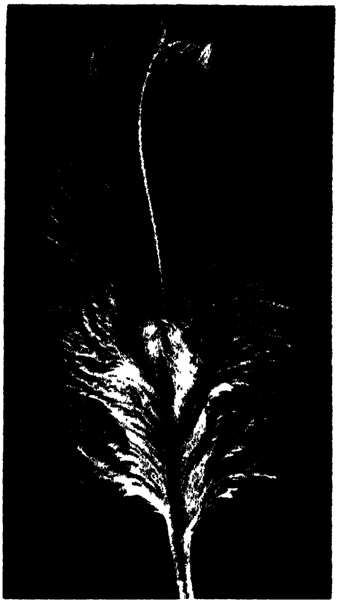
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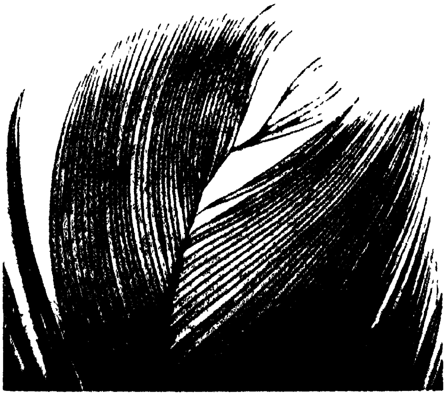
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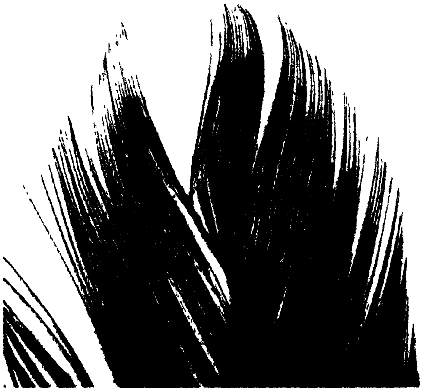
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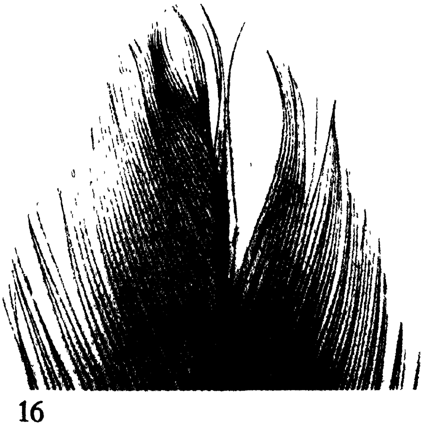
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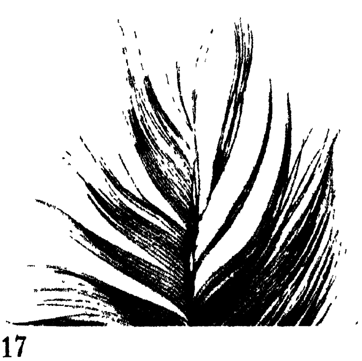
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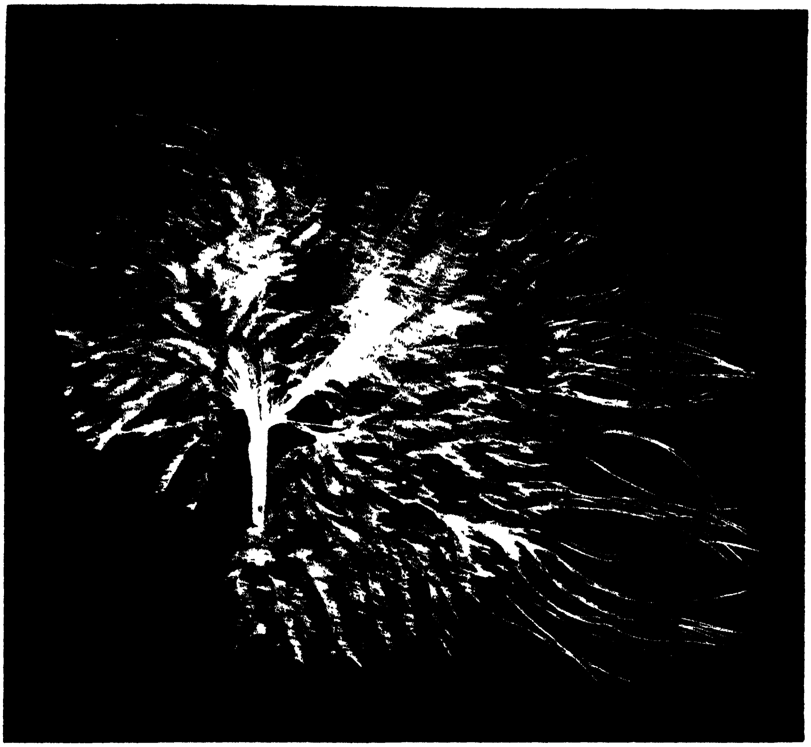
PLATE IV



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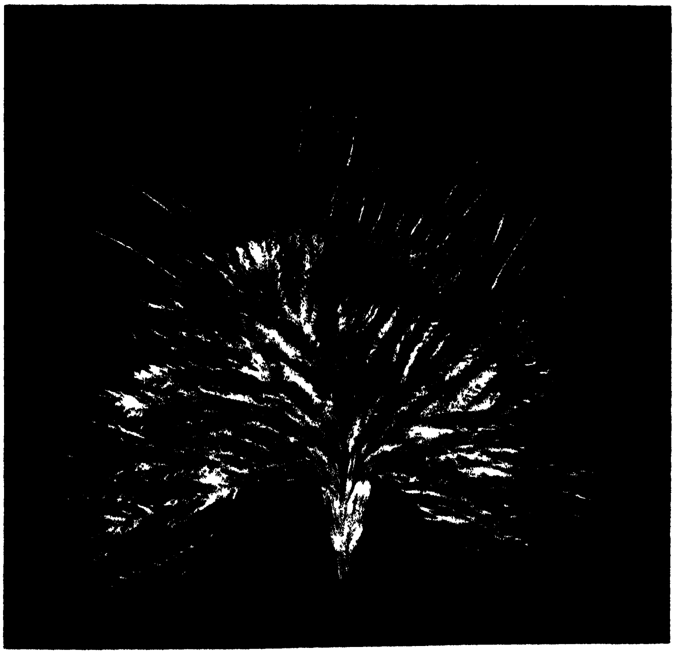

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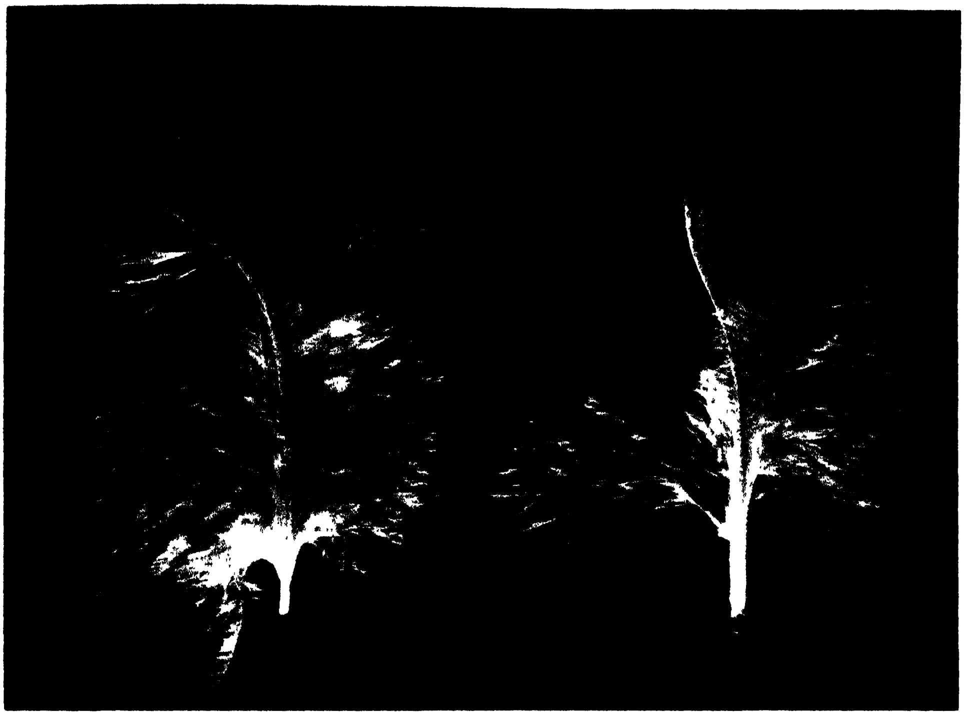

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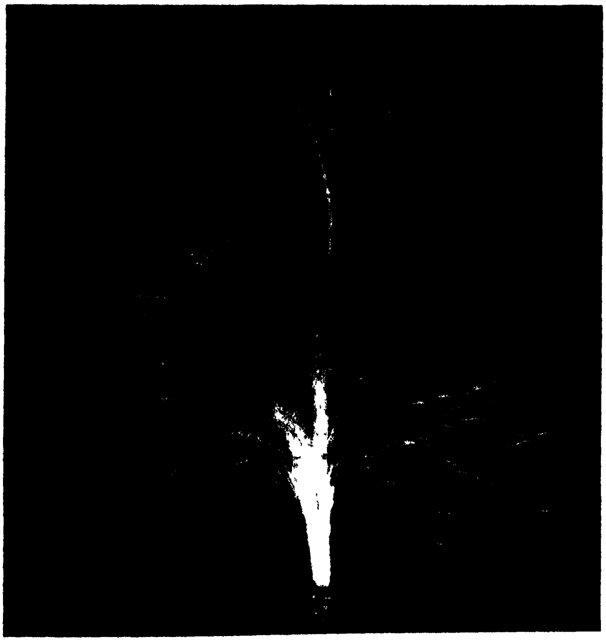

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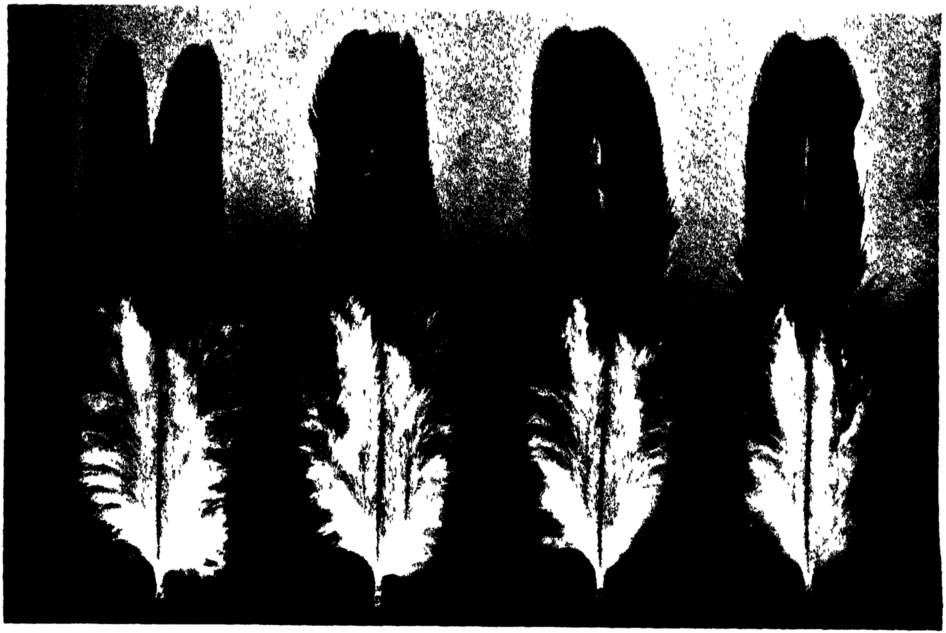
PLATE VI



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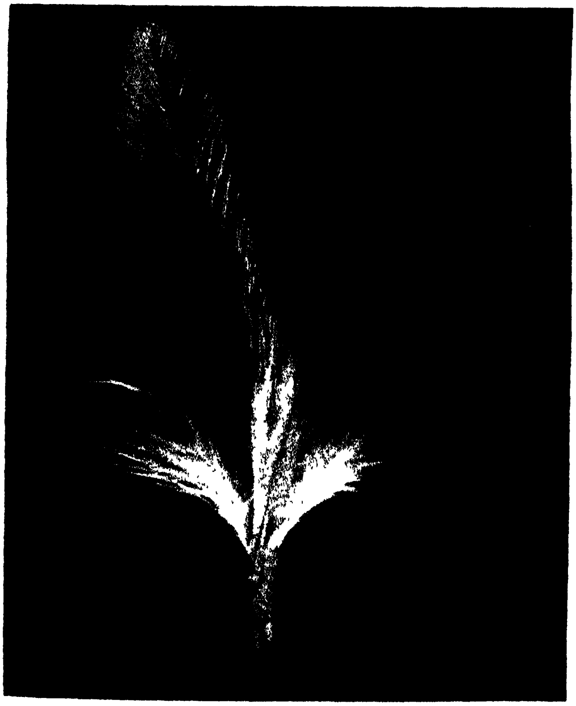

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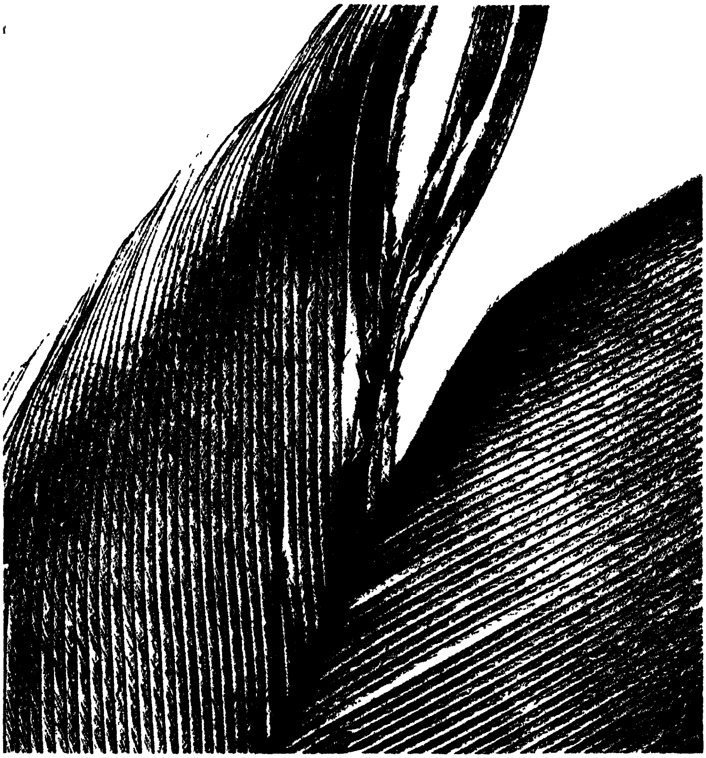
PLATE VII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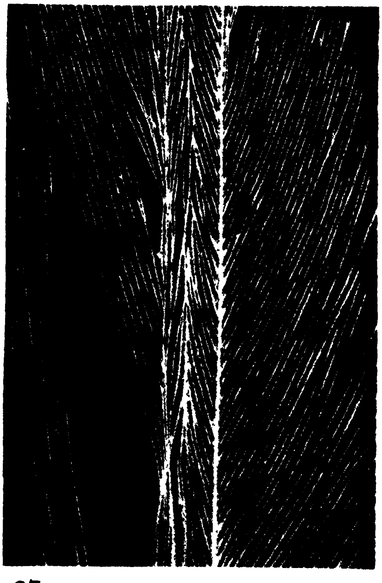
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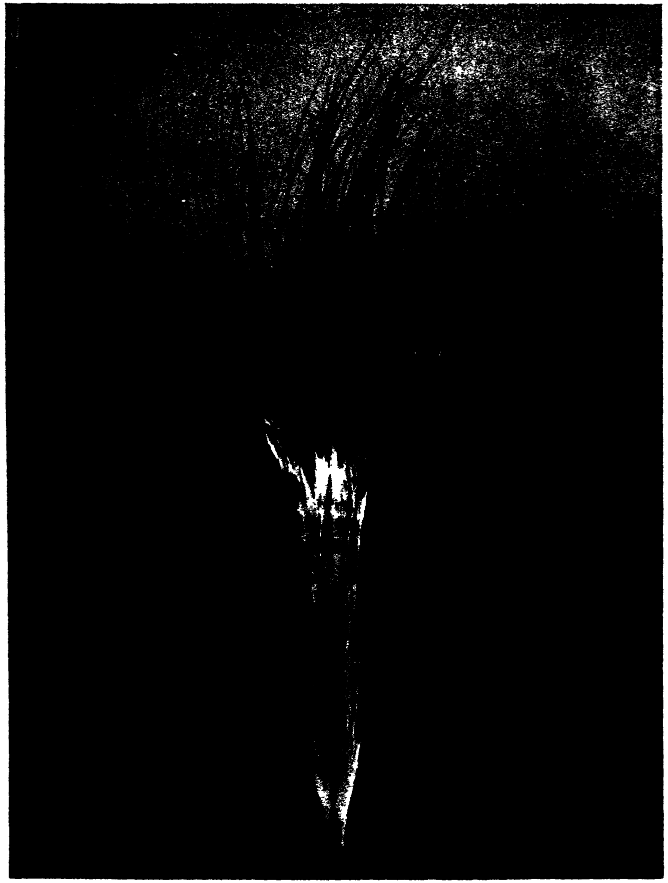
PLATE VIII



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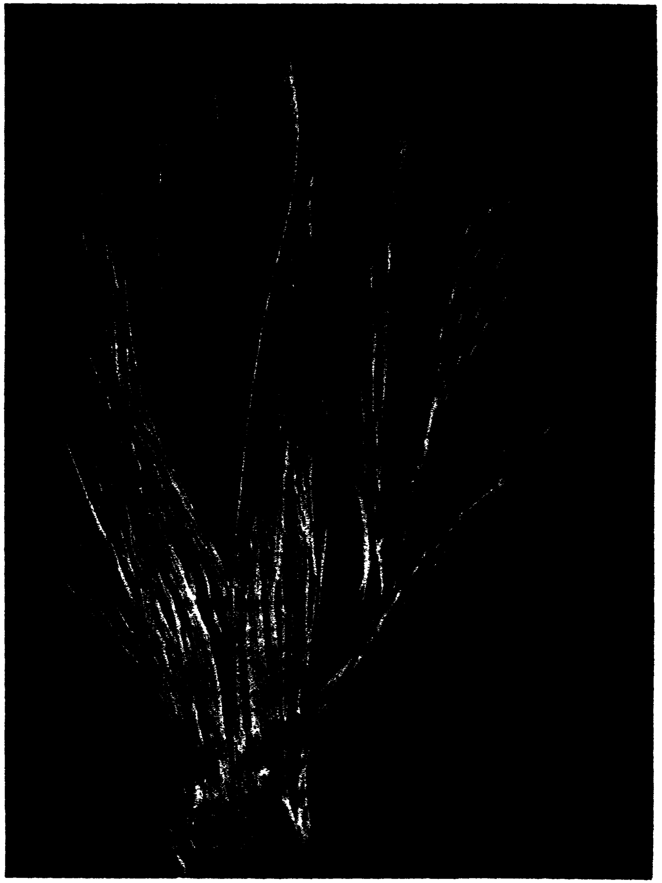

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PLATE V

FIG. 20.—First (left) and second (right) generations of a half-vane feather from Experiment F.B.D. in which the entire dorsal half of a papilla was removed, Brown Leghorn hen, breast; right side dominant; dorsal view; age of feather, 59 days for first generation, 48 days for second generation. Natural size.

FIG. 21.—Half-vane feather from Experiment F.A.D. in which the dorsal half of the apical portion of a papilla was removed, Brown Leghorn hen, breast; dorsal view; left side dominant; age of feather, 46 days post operation. Natural size.

PLATE VI

FIG. 22.—Apical twinning. Left to right: first to fourth generations from the same follicle from a sagittal isolation experiment, Brown Leghorn capon, breast. Dorsal view; age of feathers, 66 days for first generation, 72 days for second generation, 58 days for third generation, 65 days for fourth generation, post operation (see text, p. 121, for description). $\times \frac{5}{6}$.

FIG. 23.—Twin from a sagittal isolation experiment, White Leghorn hen, breast; dorsal view; age of feather, 40 days post operation. $\times 1\frac{2}{5}$.

FIG. 24.—Two pairs of twins photographed on the living bird, from a sagittal isolation experiment, Brown Leghorn capon, breast. Both pairs regenerated in a single follicle; in the one on the left each part has its own separate sheath; in the one on the right there is one common sheath for both; dorsal view; age of feather, 39 days post operation. Natural size.

PLATE VII

FIG. 25.—Apex of feather from a sagittal isolation experiment, White Leghorn hen, breast. Its apical defects consist of missing barbs, a crossing of two barbs (lower left), and bundling and branching of barbs near tip. Ventral view; age of feather, 47 days post operation; length of entire feather, 7.2 cm. $\times 6$.

FIG. 26.—Enlarged apex of feather from Experiment T, Brown Leghorn hen, breast. It illustrates apical defects consisting of union (on the right side), branching and bundling of barbs (on the left side). Dorsal view; age of feather, 59 days post operation; length of entire feather, 8.0 cm. $\times 5$.

FIG. 27.—Portion of a feather from Experiment T in which the entire apical portion of a papilla was removed, Brown Leghorn hen, breast. It illustrates on the left side a highly complicated pattern of abnormalities consisting of branching, union, and bundling of barbs producing simulation of a rhachis. Ventral view; half-vane at right is dominant; age of feather, 59 days post operation; length of entire feather, 5.2 cm. $\times 5$.

PLATE VIII

FIG. 28.—Tuft feather with basal rhachis, from F.B.D. operation on a resting papilla, Brown Leghorn male (same experiment from which the feather shown in Pl. IV [Fig. 19] is derived). Dorsal view; age of feather, 73 days post operation. $\times 1\frac{1}{2}$.

FIG. 29.—Exceptional form of half-vane feather with the feather cylinder split flat, from an isolation experiment in which a breast papilla was divided into four approximately equal axial parts, White Leghorn male. Its derivation from the parts is not known. It illustrates bundling of barbs along an isochrone which extends through both the dominant and the defective half-vanes. Note slender attachments of much-branched barbs on the right. Age of feather, 40 days post operation; dorsal view. $\times 3$.

MANIFESTATIONS OF INCOMPATIBILITY IN LIMB GRAFTS MADE BETWEEN BIRD EMBRYOS OF DIFFERENT SPECIES[1]

(Two plates, twelve figures)

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MANIFESTATIONS of host-graft incompatibility in heteroplastic transplants in Amphibia have been described by Hewitt (1934), Schwind (1937), Twitty (1937), Humphrey and Burns (1939), and others, while Loeb and other investigators have studied the problem in mammals. With the exception of several investigations made by the use of the chorio-allantoic technique, similar studies have not been reported in birds. Apparently Rawles (1938) was the first to make heteroplastic skin transplants to the body wall of avian embryos. She found on transplanting epidermis of robin embryos to the wing-bud region of embryonic chicks that the feathers produced at the site of the graft were of host type but colored like the donor. No evidence of an incompatibility was reported in this study. The author (1939*a*) described the successful transplantation of a chick limb bud to an embryonic turkey host and later made reciprocal transplants between various species of bird embryos (Eastlick, 1939*b*, 1939*c*). No macroscopic evidence of host-graft incompatibility was observed in hosts which were killed before the time of hatching. An incompatibility was observed, however, in all cases in which the hosts survived two or more weeks beyond the time of hatching. It is the object of this paper to describe macroscopic evidences of a reaction syndrome observed in duck, guinea fowl, chukar partridge, and quail appendages attached to chick hosts. For a preliminary report see Eastlick (1939*d*).

MATERIAL AND METHODS

The chicken, chukar partridge, and quail eggs were obtained at the poultry farms of the University of Missouri,[3] while the guinea-fowl and duck eggs were obtained from various hatcheries. White Leghorn and Barred Plymouth Rock chick embryos were used as hosts; the ducks were Wild Mallard and White Pekin, and the guinea fowls were of the Pearl variety.

Donor and host embryos usually were 2½–3½ days old (26–38 somites), although some younger and a few older stages were used. The method of making the transplant was a modification of the method developed by Hamburger (1933, 1938). With the removal of both the wing and the posterior limb buds four grafts could be made from a

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[2] National Research Council Fellow at the University of Chicago. The author acknowledges courtesies of the Department of Zoölogy and advice of Dr. Paul Weiss in the preparation of the manuscript.

[3] The author wishes to thank Mr. James Forward, superintendent of the poultry farms at the University of Missouri, for supplying the game-bird eggs and rendering many other favors.

single donor embryo. Limb buds left in warm, sterile physiological saline solution for an hour or more were not injured, since the fourth transplant made from a donor seemed as viable as the first. Most of the transplants were placed on the host in normal orientation, although in some cases the anteroposterior or the dorsoventral axis was reversed.

GENERAL RESULTS

Considerable difficulty was encountered in getting the hosts to survive the latter part of the incubation period. This high mortality between 19 and 21 days of incubation seems to be associated with the failure of the yolk sac to retract properly owing to malposition of the host and the membranes sticking to the shell. A few of the embryos which hatched with the yolk sac incompletely retracted were saved by tucking the ectopic sac into the coelom and suturing the umbilicus.

The grafts, for the most part, were attached to the right flank of the host just ventral and posterior to the right wing, although two cases were secured in which the transplant became fused to the wing. Also in two additional hosts the right wing was suppressed completely, and the transplant seemed to have taken its place.

The extent of attachment between graft and host varied greatly. Occasionally, when the entire proximal portion of the graft was fused to the host, a considerable area of the surrounding tissue seemed to be of donor origin as diagnosed by the difference in color of donor and host skin. In other instances a rather narrow bridge of tissue held the graft in place and a small area at the base seemed to be of donor origin.

THE INCOMPATIBILITY REACTION

I. DUCK GRAFTS

Of the fifteen cases secured, four bore well-formed duck legs, two had normal-shaped wing grafts, and the others had atypical wings. The grafts appeared healthy for a period ranging from 10 to 14 days after the hatching of the host, during which time a well-formed leg graft grew approximately twice as fast as the host's own leg (Fig. 1). This seems to be in accordance with the normally faster growth rate of the duck. The first indication of incompatibility in a transplanted leg was a slight swelling of the toes and the web. The edema then proceeded proximad over the graft, and within a day or more the basal portion had enlarged noticeably. The swelling continued so that within a few days the web became several times its normal thickness (Fig. 3). The skin of the graft soon was so tightly stretched that it took on a glossy appearance and ruptured in a number of places. Localized hemorrhages occurred. The epidermis at the edges of the cracks frequently thickened, forming a scab. Some sloughing of the epidermis was observed, especially on the web.

The feathers of the graft were injured during the period of swelling, for they ceased growing, became necrotic, and fell from their follicles, first in spots, later throughout the graft (Figs. 3, 4, and 5). The rest of the graft kept on growing rapidly. Its distal portion usually dragged on the floor of the cage, and the host stepped on it. Mash and debris clung to the moist surface of the leg and, within a week after the onset of the reaction, the foot and shank became covered with a heavy incrustation composed of scab and extraneous matter. Although pieces of the crust 1 cm. square or larger occasionally sloughed with exposure of the phalanges or bones of the shank, a scab was reformed, and healing occurred. Regressive changes took place in the ankle joints of the transplants; they gradually underwent ankylosis and eventually became entirely immobilized. The knee joints, however, remained movable throughout the life of the grafts.

Meanwhile, the donor tissue connecting the graft with the host also passed through a series of necrotic changes. Ordinarily, the place of fusion between graft and host could be seen clearly, since the duck skin had a yellowish appearance while the chick skin was greyish or white. At the beginning of the reaction in this area the donor skin became edematous. Some blisters developed, filled with fluid, and ruptured. Considerable areas of the epidermis sloughed, exposing the underlying dermis. The peripheral blood vessels in the area enlarged, and frequently fluid escaped. In some cases a sticky, yellow exudate formed, covering the skin around the base of the graft. The feathers within the affected area became dry and brittle and frequently fell from their follicles, but in no case did the reaction spread beyond the limits of the donor tissue. The grafts passed through the most drastic period within four weeks and by the end of this time were covered, entirely or in part, with a scab (Figs. 4 and 5). Healing then followed over a period of several weeks (Figs. 2 and 6). The final result of this phase was variable as illustrated by the following case histories.

Host DC 27 possessed a well-formed Mallard leg with the exception of a missing fourth toe (Fig. 1). Healing took place on the proximal portion of the transplant and gradually extended distally. The terminal end of the foot bore a scab during the eighth week (Fig. 2), but at the end of the ninth week the graft was completely healed. During the course of healing an abundance of scar tissue was formed between the toes so that the web was obliterated and a club-shaped foot resulted. At the time of autopsy in the thirteenth month, this graft still appeared healthy.

Host DC 111 possessed a wing graft which became completely covered with a scab. As this began to recede during the fifth week, the graft started to grow excessively; and at ten weeks, when the host died of coccidiosis, the wing was 33 mm. wide at the elbow and apparently had not yet reached its maximum size.

DC 129 (Fig. 5) bore a slightly atypical leg which was healing up to the seventh week when the host amputated the foot. The remaining portion of the leg then began to decrease in size, and when the bird was sacrificed in the sixteenth week the graft had almost disappeared.

DC 125 bore a leg from which the foot was missing. The graft underwent the usual reaction; but recovery occurred, and at the eighth week only the terminal end of the shank bore a scab. Host feathers grew down over the proximal portion of the leg from the base of the graft. The transplant appeared healthy up to the twelfth week, when it underwent a second reaction at which time intense swelling occurred in the distal end followed by edema in the entire leg. The skin became so tightly stretched that it ruptured. Considerable loss of fluids occurred, which matted the feathers. When they were clipped loose, the skin sloughed in sheets with the exertion of a slight tension. The surface of the leg became greenish in color, and a foul odor emanated from the graft. Fibrosis seemed to occur, as the graft was hard to the touch. By the fifteenth week the graft was decreasing in size owing to resorption and was also being cut free from the body of the host by a constriction. Since it appeared that the transplant would soon be severed, the host was sacrificed and tissue fixed for histological studies.

The fate of a duck graft following the incompatibility reaction seems to be very diverse, for it may (1) heal, cease growing, and persist as a small appendage; (2) grow abnormally large; (3) be resorbed; or (4) recover temporarily and then undergo a second reaction in which it is destroyed.

None of the hosts died or became seriously ill as a consequence of the incompatibility reaction. Well-developed grafts did cause the hosts considerable difficulty in locomotion, and in those instances in which the grafts underwent a second reaction the hosts were distinctly uncomfortable, a droopy appearance was assumed, and the furnishings of the head lost color.

II. CHUKAR PARTRIDGE GRAFTS

Six hosts hatched and reached maturity. Three bore well-formed leg grafts; one had a normal-shaped wing, while the other two possessed atypical wings. The incompatibility reaction occurred between 9 and 14 days after hatching, the onset being on the ninth day in two cases, the eleventh in one, and the fourteenth day in the others.

Two cases which illustrate the reaction syndrome will be described. Host X 29 bore a well-formed leg attached under the right wing and carried so that the host could not step on it. The feathers of the transplant were only partially colored, apparently because of the fact that many of the graft melanophores had migrated into the right wing of the host (Fig. 7). The region of fusion of graft and host tissues was indicated clearly by the dark color of the chukar partridge skin. On the ninth day the scales of the toes were dry, and some desquamation took place (Fig. 7), although no intense reaction occurred until the fourteenth day when the skin became edematous. Rupture of the skin at the ankle and at other places was followed by extravasation of fluids. Irregular scabs then formed on the distal portions of the leg and foot (Fig. 8). The graft increased in size for four weeks (Fig. 8), then was slowly resorbed; the destruction became complete when the host was 4½ months old.

The feathers, seemingly of donor origin, at the base of the graft became matted in the first few days of the reaction. They were unable to survive, and by the seventh week the graft was entirely bare. During the eighth week host feathers began to appear on the denuded regions. The new feathers were uncolored, although the original ones had been pigmented. Apparently the chukar melanophores located in the donor skin were destroyed during the tissue reaction. On the other hand, the chukar pigment cells which had migrated into the wing of the host were not injured, since they continued to supply melanin to the wing feathers of the host.

Host X 69 bore a well-formed chukar partridge wing in place of the right wing of the host. The onset of the reaction occurred on the fourteenth day. The wing became very swollen, the skin ruptured, and rather severe hemorrhages took place. The primary feathers, which had grown up to this time, became swollen at their bases and soon dried (Fig. 9). Within a week the entire wing was necrotic and was gradually pinched off at its base.

Apparently these two grafts were eliminated by quite different procedures; in one case the graft was slowly resorbed, while the other underwent necrosis and was severed from the host by a progressive constriction at the base of the transplant.

III. QUAIL GRAFTS

Of the seven cases secured, four possessed normal legs, and the others had atypical wing grafts. The grafts became slightly edematous 7–10 days after hatching, and necrosis soon followed. The rate of breakdown was rapid, and the grafts were soon eliminated. In other respects the syndrome in quail grafts seemed to parallel that observed in chukar partridge transplants.

IV. GUINEA-FOWL GRAFTS

Nine hosts were secured, five with leg and four with wing grafts. Four of the leg transplants were of normal shape, while the rest of the transplants were atypical. The onset of the reaction occurred soon after hatching, being at one day in one instance, two days in another, and on the fifth day in the rest. The first indication of the reaction was a slight swelling of the toes soon followed by edema throughout the graft. Within a day after the onset the toes began to turn black, to shrivel, and the foot fell off (Fig. 10). Next the region just proximal to the terminal end became necrotic, constricted, and broke off (Fig. 11). In this way some grafts became reduced in length until only a basal portion remained (Fig. 12). At a later date even this was lost by a constriction at the junction between host and transplant.

In some instances the entire graft became shriveled within a week after the onset of the reaction. Transplants of this type seemed to lose their blood supply and degenerated *in toto*. The feathers on the graft and skin immediately surrounding the point of attachment appeared dry and unhealthy from the time of hatching and soon fell from their follicles. The guinea-fowl skin at the base of the graft became dry and scaly, and large flakes were shed until all the donor epidermis was eliminated or replaced by host skin. Guinea-fowl grafts seldom became so much swollen that the skin ruptured, consequently there was little extravasation of plasma or hemorrhage. Moreover, these grafts were never observed to develop scabs or to heal.

DISCUSSION

The results show that limb-bud transplants may be made successfully between embryos of different species, even between embryos belonging to different orders. The grafts may undergo normal morphogenesis and appear healthy during the embryonic period, but evidence of an incompatibility occurs in varying periods after hatching. The onset of the reaction seems to occur at a definite time in each species: the interval between hatching and the reaction is 10–14 days in duck and chukar partridge grafts, 7–10 days in quail grafts, and around 5 days in guinea-fowl grafts. The rapidity of breakdown of the transplants follows a similar order, that is, the guinea-fowl grafts were eliminated in the shortest time.

While the reactions vary in detail in the different species and even in individual cases within a given group, the syndrome seems to affect commonly the blood vessels and the skin. There was an initial increased supply of blood to the affected regions as indicated by the dilation of the cutaneous vessels and the swelling of the digits. Stasis followed by hemorrhage and extravasation occurred in all cases, but to a much less extent in guinea-fowl grafts. Rupture of the peripheral capillary bed was indicated by the presence of discolored sinus-like areas in the dermis. The skin was seriously affected as indicated by edema, discoloration, scaling, and sloughing, followed by the formation of a heavy scab in all except guinea-fowl grafts. The feathers dried, became brittle, and fell from their follicles. In all cases except one of the duck grafts the final step was the destruction and elimination of the transplants. In many respects the reactions seem to resemble rather closely those found by Humphrey and Burns (1939) in heteroplastic grafts or parabiosis in Amphibia. One notable exception is that many of the amphibians died during the reaction, while none of the birds succumbed.

A number of investigators have observed the breakdown of homoplastic transplants made between birds. Roxas (1926) transplanted Leghorn testes into Sebright capons and secured a 60 per cent "take," while the reciprocal grafts were only 8 per cent successful and many of the hosts died. Juhn (1927) found that the Sebright serum seemed to hemolyze Leghorn corpuscles to a greater extent than the reverse. She considered it likely that a toxin produced by a breakdown of the Sebright tissues killed the Leghorn hosts. Danforth and Foster (1929) and Kozelka in a series of papers (1929–33) reported that a high percentage of homoplastic integumental grafts made between hatched chicks were resorbed or destroyed within 2–12 weeks after the operation. In fact, Kozelka (1929) reported that 74 per cent of his homografts were resorbed within 2 months. He observed various phases in the breakdown process, e.g., excessive vascularization, swelling, and occasional sloughing of the skin in parts of the body quite far removed from the graft. The reactions described by Kozelka do not seem as severe as those noted in the present study. This may be due in part to the larger size attained by the limb grafts and to the more distant relationship between donor and host. Kozelka (1929) found no evidence of reaction in autografts and in 1932 showed that more successful takes occurred in grafts made between closely related birds than when the relationship was distant. Loeb and Wright (1927) have suggested that the incompatibility reaction is correlated with the number of strange genes in the transplant. The author has noted evidence (unpublished data) of host-graft incompatibility in homoplastic limb transplants in the fowl. In the first group of experiments these reactions occurred during the fifth month after hatching, while in the second they began in the seventh week. The eggs in the two series were secured from different hatcheries; also they were not of the same varieties. The reaction occurred in only a few of the hosts and seemed to be somewhat different from that observed in heteroplastic transplants, although more cases will have to be secured before any general statement concerning the nature of the differences can be made.

Holman (1924) observed an intense dermatitis and desquamation following the grafting of skin of a mother to her child. He described the reaction as "similar but not identical to anaphylaxis." Recently Sandstrom and his co-workers (1939) described a fatal anaphylactoid reaction produced by transplanting macerated nephritic tissue of hatched ducklings to the chorio-allantoic membranes of embryonic chicks. The reaction was not produced by grafts of intact metanephros of hatched ducklings or by explants of embryonic tissue. Sandstrom is of the opinion that a species-specific factor of a toxic nature appears at the time of hatching and is liberated by maceration. This explanation seems plausible, since Murphy (1914) found that chick embryos became intolerant to foreign tissues between the eighteenth and the twenty-first day of embryonic life. If bird embryos do develop species specificity slightly before or at the time of hatching, why do heteroplastic grafts fail to show evidence of incompatibility immediately after hatching? A number of possibilities may be mentioned. (1) The graft may not produce incompatible proteins or toxic substances until after the hatching of the host. (2) Toxic substances may be produced, but the quantities may be insufficient to call forth the reaction. (3) The protective mechanisms of the host may not be sufficiently well developed to combat the foreign tissues until a latent period has passed. (4) The toxic substances may not be released to any extent from intact cells, and hence the reaction may be delayed until the breakdown is once initiated. (5) Grafts and host tissues may have become more or less "adjusted" to one another during the embryonic period, and this tolerance may not be lost at once. For example, it is possible that a duck limb when grown on a chick host may not possess the same degree of "duck specificity" that it would have had under normal conditions of development. The fact that the graft is isolated from other duck tissues, is nourished by metabolic products of the chick host, is in intimate contact with the host tissues, body fluids, etc., from the time when both donor and host tissues are embryonic may lead to a weakening of the tissue specificity factors in the graft. (6) The physiological age of the grafts may not be identical with their chronological age, since the transplants may be retarded because of their foreign environment. Guinea-fowl and duck embryos hatch in 28 days, while chicks hatch in 21. When this fact is taken into consideration it is obvious that the incompatibility reaction generally begins when the guinea-fowl graft is 26 days of age, or 2 days before the term of hatching. The onset of the reaction in duck grafts corresponds to a 5–7-day duckling. (7) External evidence of the incompatibility reaction may not indicate reliably the time of appearance of antibodies in the host. For example, the host may produce antibodies against duck grafts from the time of hatching, but the tissues of the transplant may be resistant and fail to show marked changes until 10–14 days later. On the other hand, guinea-fowl tissues may be more sensitive and consequently break down soon after the hatching of the host.

Hewitt (1934) has suggested that the failure of a heterograft to survive in amphibians may be due to (1) inability to use the food supplied by the host, (2) inability to excrete waste products, (3) discrepancies between the rate of growth of graft and host tissues, etc. These factors may be involved in the breakdown of heteroplastic grafts between birds.

It seems strange that a limb graft from a duck—a species which belongs to an entirely different order from that of the chick—is able to survive, while limbs from species within the same order as that of the host are destroyed. It is possible that the duck grafts are largely replaced by host tissues, while this does not occur in the other species.

Although it has not been demonstrated experimentally, it seems logical to assume that the foreign proteins of the heteroplastic grafts sensitize the hosts so that defense mechanisms are brought into play. The host may respond by walling off the graft with connective tissue, by mass infiltration of white blood cells, by the production of immune bodies, or by all three. A study will be made in an attempt to find whether or not immune reactions can be correlated with the incompatibility syndrome in heteroplastic transplants made between bird embryos.

SUMMARY AND CONCLUSIONS

1. Duck, chukar partridge, quail, and guinea-fowl limb buds were transplanted to embryonic chick hosts. Thirty-seven of the chicks hatched, and many were raised to maturity.

2. The grafts underwent an incompatibility reaction within a rather definite time after the hatching of the hosts. The time of appearance of the syndrome and its severity seem to vary in each of the species. One of the duck grafts recovered from the reaction and persisted for 13 months, while other duck appendages survived for lesser periods of time. The chukar partridge, quail, and guinea-fowl transplants did not heal following the incompatibility reaction and subsequently were eliminated.

3. The reaction seems to affect seriously the vascular system of the graft as indicated by stasis and edema. Also the donor skin ruptures, sloughs, and seems to be destroyed.
4. Probable factors underlying the syndrome are discussed.

LITERATURE CITED

DANFORTH, C. H., and FOSTER, F. 1929. Skin transplantation as a means of studying genetic and endocrine factors in the fowl. Jour. Exper. Zoöl., 52:443–70.

EASTLICK, H. L. 1939*a*. The morphogenesis of a hind limb of a 60-hour chick transplanted to a turkey embryo. Anat. Rec., Suppl., 73:17.

———. 1939*b*. Reciprocal heterotransplantation of limb primordia between duck, turkey, guinea, and chick embryos. Nature, 144:380–81.

———. 1939*c*. A study of feather character in limbs transplanted between embryos of different bird species. Proc. Natl. Acad. Sci., 25:551–57.

———. 1939*d*. Observations on species differentials in birds as studied by means of heterotransplantation of embryonic limb buds. Anat. Rec., Suppl., 75:68.

HAMBURGER, V. 1933. A method of transplanting limb buds in chick embryos. Anat. Rec., Suppl., 55:58.

———. 1938. Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. Jour. Exper. Zoöl., 77:379–98.

HEWITT, D. C. 1934. Xenoplastic transplantation of amphibian eye rudiments. Jour. Exper. Zoöl., 69:235–67.

HOLMAN, E. 1924. Protein sensitization in isoskin grafting. Surg., Gyn , and Obst., 38:100–106.

HUMPHREY, R. R., and BURNS, R. K , JR. 1939. An incompatibility manifested in heteroplastic parabiosis or grafting in Amblystoma due to a toxin of cutaneous origin. Jour. Exper. Zoöl., 81:1–42.

JUHN, M. 1927. Note on the hemolytic action of Sebright serum on Leghorn corpuscles. Biol. Bull., 52:474–79.

KOZELKA, A. W. 1929. Integumental grafting in domestic fowl; transplants of combs, spurs, and feathers in study of sex dimorphism. Jour. Hered., 20:3–14.

———. 1931. Genetic and physiological factors involved in the absorption of integumental grafts in the Leghorn fowl. Anat. Rec , Supp., 48:24.

———. 1932. Integumental grafting as a means of analyzing the factors determining the secondary sexual characters of the Leghorn fowl. Jour. Exper. Zoöl., 61:431–95.

———. 1933. Serological studies of tissue antagonism in the domestic fowl. Physiol Zool., 6:159–84.

LOEB, LEO, and WRIGHT, S. 1927. Transplantation and individuality differentials in strains of inbred rats. Amer. Jour. Path., 3:143–67.

MURPHY, J. B. 1914. Studies in tissue specificity. III. Factors of resistance to heteroplastic tissue-grafting. Jour. Exper. Med., 19:513–22.

RAWLES, M. E. 1938. The production of robin pigment in White Leghorn feather cells by skin ectoderm grafts of the robin embryo. Anat. Rec., Suppl., 72:68.

ROXAS, H. A. 1926. Gonad cross-transplantation in Sebright and Leghorn fowls. Jour. Exper. Zool., 46:63–119.

SANDSTROM, C. J.; EISEN, H. N.; and SIFFERT, R. S. 1939. Reciprocal heteroplastic chorio-allantoic transplantations of macerated chick and duck kidney tissue. Anat. Rec. Suppl., 75:120–21.

SCHWIND, J. L. 1937. Tissue reactions after homoplastic and heteroplastic transplantation of eyes in the anuran amphibia. Jour. Exper. Zoöl., 77:87–108.

TWITTY, V. C. 1937. Experiments on the phenomenon of paralysis produced by a toxin occurring in Triturus embryos. Jour. Exper. Zoöl., 76:67–104.

PLATE I

FIG. 1.—14-day White Leghorn host bearing a Mallard duck graft. Down on graft unpigmented owing to exclusion of neural-crest cells. Leg well formed with exception of missing fourth toe.

FIG. 2.—Same host as above. 8 weeks of age. Graft has healed with exception of distal end of foot. Foot is club shaped owing to scar tissue.

FIG. 3.—14-day Barred Plymouth Rock host bearing White Pekin duck leg. Host melanophores have pigmented the down on the transplant. Foot edematous, and down is becoming necrotic.

FIG. 4.—Same donor as above. Host 26 days old. Note the thick incrustation on foot and shank. The feathers have sloughed in some areas.

FIG. 5.—28-day Barred Plymouth Rock host bearing a White Pekin duck leg. Entire graft is incased in scab. Graft denuded.

FIG. 6.—Same host as in Figures 3 and 4. Host died of coccidiosis at 6 weeks of age. Note that upper portion of graft has healed.

PLATE II

FIG. 7.—9-day White Leghorn host bearing a chukar partridge leg. Donor melanophores have migrated into wing of host. Some slight desquamation of foot epidermis can be seen.

FIG. 8.—Same host as above at 28 days of age. Note the scab on foot and shank and the matted feathers surrounding the graft. Graft has reached its greatest size. Resorption took place from this time.

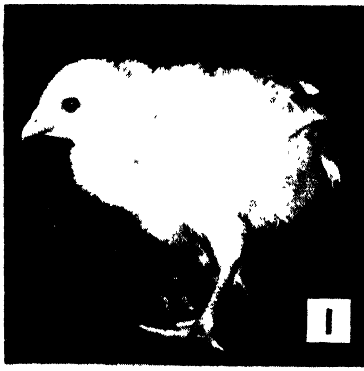
FIG. 9.—15-day White Leghorn host bearing a chukar partridge wing. Host wing suppressed. Graft necrotic. Was pinched off at base within a few weeks.

FIG. 10.—5-day White Leghorn host bearing a guinea-fowl leg. Toes of graft shriveled and necrotic.

FIG. 11.—Same host as above at 21 days of age. Foot missing. Rest of graft becoming necrotic.

FIG. 12.—Same host as above, but at 8 weeks. Entire graft "mummified." Was pinched off at base during fifteenth week.

PLATE I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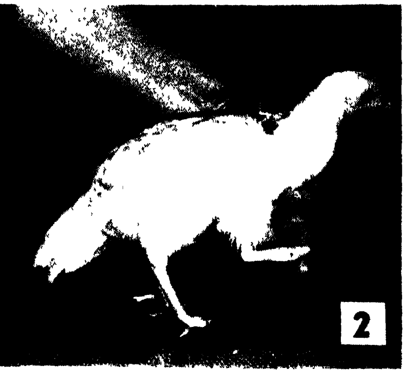
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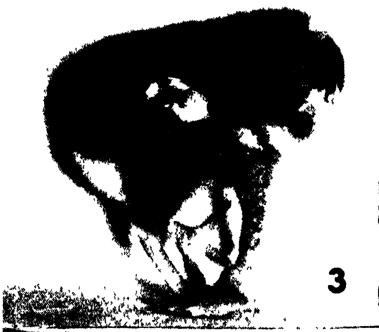
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PLATE II

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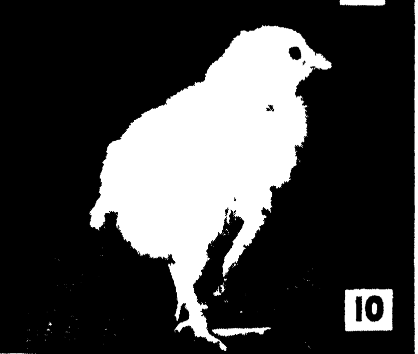
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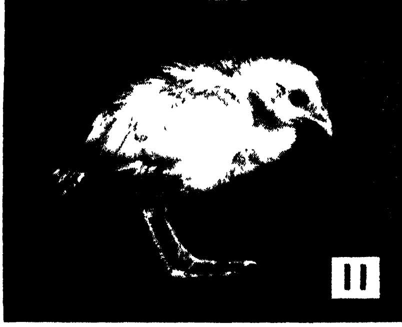
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RAT PROSTATE AND SEMINAL VESICLE GRAFTS IN RELATION TO THE AGE AND SEX OF THE HOSTS[1]

(Two plates)

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A STUDY (Price, 1936) of the results of early prepuberal castration of rats in relation to ventral prostate and seminal vesicle gland development introduced a number of problems. It was found that when rats were castrated within a few days after birth the ventral prostate continued growth, although at a slower rate, and that differentiation proceeded and the epithelium of the gland developed cell "light areas" when the rats were 17 days of age, as compared with 12 days for normal controls. These distinctive areas in the cells have been found to be constantly present in the adult functional gland, and they disappear within a few days after castration of adults. In the young castrate the light areas developed, the acini became distended, and a nearly normal histological condition was maintained until the animal was 30–40 days of age. At that time the light areas disappeared, the epithelium became flat, acini small, and the tissue typically castrate.

These findings, i.e., that the ventral prostate of the young castrated animal reached a differentiated state and maintained it for a certain limited period of time, required further analysis. The assumption was that the prostate in the young castrate differed in its behavior from that of adult castrates because of (1) some factor or factors present in the young animal and able to influence the prostate but absent in the adult or (2) an ability of the prostate to "self-differentiate." The tissue-transplantation method was used as a means of studying the problem and gaining a further understanding through prostate-transplant reactions of the hormones circulating normally in animals at various stages in the reproductive cycle. The prostate gland is well adapted as an indicator of androgenic substances because of its sensitivity, while the seminal vesicles have a higher threshold. In earlier work (Wiesner, 1934; Price, 1936) it was found that the seminal vesicles did not differentiate after early prepuberal castration but continued to grow, though to only a very limited extent. In the present study it was of interest to transplant seminal vesicles for comparison with the prostate. A preliminary report on a portion of this work was made (Price, 1937).

MATERIAL AND METHODS

Ventral prostate and seminal vesicle tissue from rats varying from 5 to over 100 days of age was transplanted into hosts ranging from 1 to 144 days of age. The transplants remained in the hosts for periods varying from 13 to 246 days. The hosts were young and old normal and castrated males, young and old normal and spayed females, and pregnant females. In all, 890 transplants were made into 316 hosts, and 335 grafts were recovered.

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[1] This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

TABLE 1

SUMMARY OF PROSTATE TRANSPLANTATIONS IN RELATION TO HOST AGE AT AUTOPSY

DONOR	HOST			TRANSPLANTATIONS										
Age in Days at Transpl.	Sex	Age in Days		No. Made	No. Rec.	% Rec.	Length of Transpl. Period	Age in Days	Histology					
		At Transpl.	At Autopsy						L.A.	Cast.	Un-dev.	Deg.	No Gl.	% +
Under 35	♂	Under 35	Under 35	18	10	55	13–29	25–54	8	. .	.	1	1	80
			Over 35	8	2	25	23	33	2	. .	...	.	..	100
	♂̸	Under 35	Under 35	37	17	46	13–20	25–54	7	2	1	5	2	41
			Over 35	18	8	44	21–123	33–131	. .	5	.	1	2	0
	♀	Under 35	Under 35	12	9	75	13–20	25–31	6		...	2	1	66
			Over 35	20	13	65	23–90	33–102	1	10	. .	2	..	7
	♀̸	Under 35	Under 35	13	7	53	17–30	30–42	3	1	. .	3	.	57
			Over 35	15	9	60	40–130	50–135	.	8	...	..	1	0
Under 35	♂	Over 35	Over 35	6	5	83	20–197	31–208	4	.		1	. .	80
	♂̸	Over 35	Over 35	60	36	60	18–80	28–91	.	...	28	7	1	0
	♀	Over 35	Over 35	40	19	47	20–245	31–253	13		1	...	5	68
	Preg. ♀	Over 35	Over 35	16	9	50	20–203	31–211	7	1	...	.	1	87
	♀̸	Over 35	Over 35	64	29	45	20–68	31–79	...	..	22	4	3	0
Total	..	. ..	.....	327	173	53	. .. .	.. .	.	...		...	.	...
Over 35	♂	Under 35	Under 35	39	7	18	13–21	89–109	2		.	4	1	28
			Over 35	4	0	0	24–28	65	.	.	..	.	. .	0
	♂̸	Under 35	Under 35	30	5	17	13–21	94–109		3	. .	2	..	0
			Over 35	5	2	40	24–35	125	. .	..	.	1	1	0
	♀	Under 35	Under 35	42	3	7	13–20	89	.	....	.	2	1	0
	♀̸	Under 35	Under 35	14	3	21	20	100	....	...	.	3	.	0
			Over 35	5	0	0	20	100	....	. ..	....	...	.	0
Over 35	♂	Over 35	Over 35	23	8	35	20–203	66–251	7	.. .		1	..	87
	♂̸	Over 35	Over 35	15	1	6	20	84	...	..	.	1	...	0
	♀	Over 35	Over 35	39	3	8	20–70	84–118	. .	1	..	2	.	0
	Preg. ♀	Over 35	Over 35	14	1	7	74	122	..	1	.	...	...	0
	♀̸	Over 35	Over 35	11	2	18	20	84	.. .	.	. .	2	.	0
Total	. .	.. . ...	.. .. .	241	35	14	. .....	....	..	. .	....	.	..	.
Grand Total	...	....	. ...	568	208	36	....	....	....	....	....	. ..	....	...

L.A. = light areas; Cast. = castrate; Undev. = undeveloped; Deg. = degenerate; No gl. = no gland tissue; %+ = percentage with light areas.

The colony of albino rats in our laboratory, although not closely inbred, gave, in general, fairly satisfactory results. Autotransplantations were done in some cases, and both littermate and nonlittermate combinations were made.

The tissue at transplantation was removed from a freshly killed donor and placed as rapidly as possible in subcutaneous sites on the abdomen of the host. At autopsy the transplants were found attached to the skin or body wall, from which they could be easily dissected for weighing. Usually 1 or 2 pieces of prostate and seminal vesicle weighing from 1 to 3 mg. were transplanted into each host. Castrations and spayings were done at the time the transplantations were made.

At autopsy of the hosts the grafts were removed, weighed, fixed in Bouin's fluid, and sectioned serially at 10 μ. In some grafts prostate cell heights and acinous diameters and seminal vesicle cell heights were measured for comparison with the host. The host reproductive tract was examined, and the ventral prostate and seminal vesicles of males were weighed, and samples were fixed for comparison with the grafts. In female hosts the ovaries were examined, and the uterine diameter measured. In spayed females an examination was made to determine that no ovarian regeneration had taken place, and the uterus was measured.

The weight of the recovered grafts varied from approximately 0.2 mg. to 123 mg. for the prostate, and from 0.4 mg. to 159 mg. for the seminal vesicle. It was not possible to predict the histological condition of such grafts from their weights. Within limits, however, one can say that very small grafts were more likely to contain only degenerate glands, fat, lymphocytes, and connective tissue; while very large grafts probably would contain more or less normal tissue, with light areas in the prostate and secretion granules in the seminal vesicles.

Table 1 presents a summary of the data on prostate grafts. The tissue was classified as under or over 35 days of age at transplantation, since 30–40 days had been shown to be a crucial age in the young, castrated rat prostate. However, since the main problem was to test out the ability of the young gland to differentiate, in the transplants listed as under 35 days of age tissue between 5 and 12 days of age was used in all but a few cases. A few transplants of tissue between 12 and 35 days of age were made. Transplants listed as over 35 days varied from 40 to about 150 days in age at transplantation.

The age of the host at the time of transplantation and autopsy was found to be the important factor in relation to the development and differentiation of prostate tissue in gonadless hosts; and in consequence the data were divided, according to the autopsy age of the host, into 35 days and under, and over 35 days.

Seminal vesicle grafts are summarized in Table 2 in the same form as the prostate grafts.

PROSTATE TRANSPLANTS

Since the interpretation of the condition of the grafts depended upon histological study, a brief review of prostate histology and the development of the gland is necessary. The ventral prostate of the rat consists of many acini which branch and coil. They lie close together (see Fig. 1) with a small amount of interacinous connective tissue and are lined by columnar epithelium in which the nuclei are basal. A distinct light band appears in the cytoplasm approximately halfway between the nucleus and the lumen end of each cell. At 5 days of age—the earliest stage at which transplants were made—the gland contains only solid cords of cells. These begin to hollow out by 10 days of age, and

TABLE 2

SUMMARY OF SEMINAL VESICLE TRANSPLANTS

Donor	Host			Transplantations									
		Age in Days					Histology						
Age in Days at Transpl.	Sex	At Transpl.	At Autopsy	No. Made	No. Rec.	% Rec.	Sec. Grs.	Cast.	Simple	Compl.	Deg.	No Gl.	% +
Under 35	♂	Under 35	Under 35	15	11	73	...	...	2	9	...	..	...
			Over 35	14	6	43	4	...	...	2	...	...	67
	⚦	Under 35	Under 35	23	8	35	..	...	6	1		1	...
			Over 35	14	4	29	..	...	4	...	...	.	...
	♀	Under 35	Under 35	12	5	42	...	...	2	3	...	..	...
			Over 35	4	1	25	...	..	1	...	...	..	...
	♀̸	Under 35	Under 35	13	3	23	...	..	3	...	...	...	..
			Over 35	5	2	40	...	...	2	...	...	..	..
Under 35.. .	♂	Over 35	Over 35	7	7	100	6	...	..	...	...	1	86
	⚦	Over 35	Over 35	14	10	71	...	...	6	3	...	1	..
	♀	Over 35	Over 35	39	18	46	...	.	8	7	2	1	..
	Preg. ♀	Over 35	Over 35	21	10	48	1	...	3	6	..	..	10
	♀̸	Over 35	Over 35	11	7	64	...	...	2	3	1	1	...
Total. . .	.....	...	... ...	192	92	48	...	...	.. .	..	.	..	.
Over 35.. ...	♂	Under 35	Under 35	16	4	25	.	..	.	..	3	1	..
			Over 35	3	0	...	..	.	...	...	.	.	..
	⚦	Under 35	Under 35	6	1	17	..	...	...	..	1	.	..
			Over 35	5	0		..	.	...	..	.	.	..
	♀	Under 35	Under 35	10	2	20	...	...	...	...	2	...	...
	♀̸	Under 35	Under 35	14	5	36	...	2	...	2	.	1	..
			Over 35	5	0	...	...	...	...	...	...	..	...
Over 35	♂	Over 35	Over 35	20	14	70	9	...	...	...	5	...	64
	⚦	Over 35	Over 35	12	1	8	...	...	1	...	...	..	...
	♀	Over 35	Over 35	21	5	19	...	..	2	..	1	2	...
	Preg. ♀	Over 35	Over 35	9	3	33	...	3	...	...	...	.	...
	♀̸	Over 35	Over 35	9	1	11	...	...	1	...	...	...	...
Total....	... ..	....	... ...	130	36	27	...	...	...	...	...	...	...
Grand total.	... ...	....	....	322	127	39	...	...	...	...	...	...	...

Sec. Grs. = secretion granules; Compl. = complex structure; %+ = percentage with secretion granules.

and at 12 days many acini have lumina, and pale light areas are present (see Fig. 8). Further development involves enlargement of the acini, increases in epithelial height and strength of light areas, and growth of villous projections in the acini. By 25 days the gland is approximately like that of the adult, except that the gross size and the acinous size are smaller.

Castration of the adult involves, first, loss of cell light areas within 4 days and then progressive reduction in epithelial height, nuclear size, and acinous diameter. The epithelium becomes pseudostratified, and the nuclei pyknotic. Interacinous connective tissue appears to increase in amount as the acini grow smaller. As has been stated above, castration of the young rat within the first week of life does not result in regression of the ventral prostate.

It was possible by histological study of the grafts, using the criteria given above, to classify the tissue as undeveloped when the acini were small, round, and usually solid; normal when light areas were found in any of the acini; castrate; and degenerate. The condition of the acini in different parts of a graft frequently varied greatly. In some grafts the relation of the acini was normal (see Fig. 1), but in others less so (see Fig. 2), and in still others groups of acini were scattered in large amounts of connective tissue. Acinous size varied, and normal, quite distended acini containing epithelial light areas were often closely associated in the same graft with others that were small and castrate in appearance. Lymphocytes were usually present. Connective tissue was frequently abundant. In some grafts, particularly of older tissue, it completely obliterated the lumina of prostate acini and seminal vesicles. All these changes were observed in tissue from all classes of hosts and resulted from the abnormal conditions that necessarily obtain in tissue placed in a foreign environment and growing under the influence of foreign host reactions and changed vascularity. In spite of the somewhat abnormal appearance of many of the grafts, it was a relatively simple matter, in most cases, to determine the degree of development or regression.

A. TRANSPLANTS OF PROSTATE TISSUE UNDER 35 DAYS OF AGE AT TRANSPLANTATION

1. *Into young and old normal males.*—A few transplants of young prostates were made into normal males to test the ability of the tissue to survive and develop normally. A relatively high percentage of grafts was recovered (see Table 1), and, of these, 80–100 per cent possessed light areas in the epithelium. The histological character of the transplants varied from grafts that appeared normal to others that had only a few scattered acini and were in almost castrate condition. In some only a few acini showed light areas. It is true, however, that in normal prostate glands which are responding to a low male hormone level, as when castrates are injected with low dosages of male hormone, the acini enlarge from the castrate state; but frequently the peripheral acini are more nearly normal histologically than are other parts of the gland and have light areas when none can be found elsewhere. Thus there is, even in the intact gland, a differential response of the acini, seemingly associated with position in the gland.

Six transplants were made into older normal males, over 35 days of age at transplantation time; 5 were recovered, and 4 of these, or 80 per cent, had light areas. These grafts all showed great distention of the acini, greatly stretched and flattened epithelium, and large amounts of secretion. There was, of course, no outlet for secretion in the grafts; and the resulting accumulation of secretion and pressure made the epithleium, in some cases, flat, vacuolated, and abnormal. This factor of distention was found to be operating in grafts recovered from old normal males, some old normal females, and several pregnant females and appears to indicate a large amount of androgenic stimulation. The weights of the grafts, although not always dependable, do give additional support here to such a theory. The best positive grafts from older males weighed about 40 mg., as against 3.6 mg. for the best grafts from young males. Light areas were maintained in grafts for periods of a little over 6 months and could undoubtedly have been maintained longer.

2. *Into young and old castrated males.*—Approximately half of the transplants put into young castrated males survived, but several were degenerate or had no gland tissue. The grafts recovered from hosts over 35 days of age at autopsy differed from those recovered from younger hosts and will be treated separately.

Seven of the 17 grafts recovered from younger graft hosts had light areas. The best is illustrated in Figure 4. This tissue was transplanted from a 10-day-old male into a 5-day-old male (castrated when the transplant was made) and allowed to remain for 20 days, so that the host was 25 days old at autopsy and the graft tissue 30 days. The acini were distended, the epithelium moderately high, and light areas were somewhat pale but present consistently in many of the cells. A comparison of Figure 4 with Figure 3—the prostate of the host from which the graft was taken—shows that graft and host prostate were about equally well developed. Neither equals the development of the normal prostate of a 25- or 30-day-old normal male. The remaining 6 grafts were not so well differentiated, and, in some, very pale light areas were found in only a few cells. Two of the recovered grafts were definitely castrate in appearance, and 1 graft was classified as undeveloped and had small acini of a type that will be discussed later. It must be stressed that the prostates of young castrated males do not all show the same degree of differentiation; and they vary, at the age of 30 days, from an almost normal state to nearly castrate and in some few cases to a completely castrate one. The grafts show the same variability as the prostates of castrate males. Measurements of cell heights and acinous diameters showed that, in all but the examples shown in Figures 3 and 4, the acini of host prostates were more distended than those of the grafts and host epithelium was higher. The longest period of maintenance of cell light areas in castrated hosts was 20 days, when the host was 30 days old and the graft-tissue age was 34 days.

Young prostate transplants recovered from young castrates over 35 days of age when killed proved to be in a castrate state at that time, like the host prostate. Figure 5, the host prostate, and Figure 6, the graft, illustrate these findings. An autotransplant was made into an 11-day-old castrated host and left for 45 days. Host and graft were 56 days old when the host was killed. As Figures 5 and 6 show, both prostates were in typically castrate condition, with small acini and flat epithelium. Five of the 8 grafts in this category were like this, while 3 were degenerate or had no gland tissue. The number of grafts here is low, but grafts from the same-age spayed female and normal female hosts (usually under 80 days of age) gave the same results. One can conclude that grafts from castrated males killed below 35 days of age will have light areas and at least approach a normal functional state, while those from castrated hosts over 35 days of age at autopsy will show a castrate condition.

Some of the most interesting results were found in transplants of young prostate gland put into castrated males over 35 days of age at transplantation time. The grafts remained in the hosts for periods varying from 18 to 50 days. Twenty-eight of the 36 recovered grafts contained only small, undeveloped acini of the type shown in Figure 7. Ten-day-old prostate was put into a 47-day-old castrated male and allowed to remain for 18 days. The graft tissue in Figure 7 was 28 days old at autopsy, and the host 65 days old. The host prostate is not shown, but it was approximately like that in Figure 5 (a castrated male at 56 days) and was a typical castrate. The graft acini in Figure 7 can be seen to be relatively normal in appearance, with no indications of degeneration, and lymphocytes are not especially in evidence. The tissue resembles the normal young prostate at about 11 days of age (Fig. 8), except that some regression is evident as shown by the flat epithelium and small nuclei. A comparison of Figure 7 with Figure 6 shows the difference between tissue that has never developed to any marked degree and tissue that has developed as in Figure 4 and ultimately regressed. In some of the other 28 grafts the acini were smaller than those shown in Figure 7, and frequently all the acini were solid. Again, these 28 grafts varied, and some contained only a few acini, but these never were moderately or widely distended or had any indications of light areas. The graft in Figure 7 weighed 2.0 mg. as compared with 5.0 mg. for the graft shown in Figure 4 and 2.1 mg. for the graft in Figure 6. The 8 remaining grafts that did not show this type of acini were definitely degenerate.

3. *Into young and old normal females.*—Prostate transplants in young normal females killed at less than 35 days of age had light areas in 6 out of 9 recovered grafts. The remainder were degenerate. These 6 grafts varied as did all groups of grafts; but, in general, the range of acinous diameters and cell heights was about like that found in grafts from young normal males, and the grafts differed in no way histologically from grafts from males. The light areas seemed stronger and more consistent than those observed in grafts from young castrated males.

Transplants taken from females over 35 days of age at autopsy presented very different results, with the exception of 1 case. Ten of the grafts were typically castrate histologically, 1 had light areas, and 2 were degenerate. These findings were in accord with those found in castrated male hosts killed over 35 days of age. However, the female presents a more complicated situation. The 10 grafts that were typically castrate were recovered from hosts that were between 52 and 79 days old at autopsy, while the 1 containing distended acini, moderately high epithelium, and pale light areas was recovered from a 102-day-old female and had been transplanted for 90 days.

This difference in character of grafts from older females was still more marked in the grafts put into females over 35 days old at time of transplantation. Of 19 recovered grafts, 13 had light areas, 1 was undeveloped, and 5 had no gland tissue. Two of the positive grafts were taken from the same 71-day-old host, but all the other positive cases were from hosts ranging between 97 and 387 days of age at autopsy. The indications are that grafts from young females under 35 days of age will contain light areas in a high proportion of cases; from females between 35 and approximately 80 days of age the grafts will be mainly castrate, although an occasional female near the upper end of the age range may yield a positive graft (as indicated by 2 positive grafts from a 71-day-old female); from females over 80 or 90 days old the grafts will again contain light areas. The possible significance of this will be discussed later.

The graft in Figure 2 is typical of the 13 positive grafts obtained from older females. The tissue was transplanted at 15 days into a 141-day-old female host. After 42 days the graft was recovered, at 57 days of age, with the host 183 days old. The acini were somewhat scattered, but they appeared quite normal, and light areas were well marked. Grafts of young prostate tissue into older females were recovered approximately 8 months later and found to contain light areas. The largest graft in this group weighed 32.8 mg., which compares favorably with the largest from old normal males.

4. *Into pregnant females.*—Prostate transplants were also put into females that were already pregnant or were bred after transplantation of the tissue. When these females littered they were bred again, so that, for example, one female carrying a graft littered 7 times in 7 months. Nine grafts were taken from such females after periods ranging from 20 to 203 days. Seven of the grafts could be classified as appearing functional and possessing light areas, 1 was castrate, and 1 had only connective tissue. Several of the grafts had greatly distended acini and flat epithelium, as described above for grafts from old normal males, but others had more nearly the usual distention of acini, and the graft shown in Figure 1 is one of these. Eleven-day-old tissue was transplanted into a 68-day-old female that littered twice during the 68 days she was carrying the graft and was found to be pregnant again at autopsy. The host was 136 days old when killed, and the graft tissue was 79 days old. The graft weighed 52 mg. and was indistinguishable histologically from the prostate of a normal male except that there were no villi in these acini.

The grafts from pregnant females showed marked androgenic stimulation not only in the great acinous distention but also in the gross size of several of the grafts. The largest graft from old normal males weighed 39.8 mg. (unfortunately, not all grafts were weighed); from old females, 32.8 mg.; and the 3 largest from pregnant females, 33.4, 52.4, and 123 mg. The last is the largest prostate graft recovered in the course of the experiments. No estrogenic effects were observed in any prostate grafts recovered from normal young or old females or pregnant females.

5. *Into young and old spayed females.*—The transplants of tissue in these categories were found to duplicate the findings in young and old castrated male hosts. Young tissue put into young spayed female hosts killed under 35 days of age resulted in 7 grafts, 3 of which had light areas, 1 was castrate, and 3 were degenerate. In general, the best positive graft was similar to the graft in Figure 4 from a castrated male host. The longest maintenance of light areas was for a period of 30 days in tissue transplanted from a 10-day-old male to a 5-day-old spayed female. The host was 35 days old at autopsy, and the graft 40 days old. When the hosts were over 35 days at autopsy age, 8 castrate grafts were recovered, and 1 with no gland. The 8 grafts were typically castrate and resembled Figure 6.

Prostate grafts in older spayed females yielded the same result as in older castrated males. Twenty-two out of 29 grafts contained small acini like those shown in Figure 7.

B. TRANSPLANTS OF PROSTATE TISSUE OVER 35 DAYS OF AGE AT TRANSPLANTATION

It was found that prostate tissue from older rats was very limited in its ability to survive. Only 14 per cent of the transplants were recovered in comparison with 53 per cent when young tissue was used. The recovered grafts proved to be degenerate in 51 per cent of the cases, while only 15 per cent of the grafts of young tissue were degenerate.

The transplants of older prostate tissue were taken from males varying in age between 38 and 150 days. The prostates at the time of transplantation were, of course, well developed, and the problem was one of maintaining the differentiated condition.

When older prostate transplants were put into young hosts, a small number of grafts was recovered, as shown in the lower half of Table 1. It proved very difficult to obtain takes even in young males. Thirty-nine such transplants yielded only 7 grafts, and 2 of those had light areas, while 5 could be considered degenerate and lacking gland tissue. These 2 positive grafts were from the same host and were in approximately the same histological condition with only a few acini, and some of these were abnormal, some castrate, and a few had pale light areas. The host was 29 days old at autopsy, the tissue 89 days, and the transplantation period 20 days. From young castrated males killed under 35 days of age, only 5 grafts were recovered, and 3 were definitely castrate. In young castrated males killed over 35 days of age, in normal females, and in spayed females only a small percentage of grafts was found; and these recovered grafts were all so degenerate that no conclusions can be drawn as to whether old prostate tissue might be kept in a functional state in such hosts. The same holds true for all classes of old hosts except old normal males, in which 8 grafts were recovered from 27 transplants—a recovery of 35 per cent. Seven of these grafts were classified as functionally normal although some were nearly castrate. Others had moderately distended or widely distended acini with light areas. The graft weights in old normal males indicated stimulation, for they ranged from 4 mg. to 8 mg. Grafts in other hosts gave weights from 0.4 mg. to 3.4 mg., with 50 per cent of them weighing less than 1 mg.

SEMINAL VESICLE TRANSPLANTS

The seminal vesicles of the adult rat are paired structures containing a central lumen which runs the length of each gland and gives off ramified and complicated side branches. The epithelium is tall columnar with basal nuclei, and the cytoplasm contains prominent secretion granules surrounded by a clear area of cytoplasm or "halo."

The development of the seminal vesicle differs from that of the prostate in that it does not reach a differentiated state, as judged by the presence of secretion granules in the cells, until about 36 days of age, while the light areas of the prostate cells can be found at 11 or 12 days of age. At 5 days of age the histological structure of the seminal vesicle is very simple, for it consists of a central lumen from which small side branches are just beginning to grow. By 15 days the lateral growths are very complex, and at 30 days this is still more apparent, and the cells have become highly granular. The thirty-sixth day marks the beginning of true secretion granules in the cells, which by this age have increased to tall columnar. Further development includes great and rapid increases in gross size, cell height, and number and size of secretion granules.

Castration when the gland is in its adult, differentiated condition results in loss of the secretion granules within 48 hours and subsequent reduction in cell height, degenerative changes in the nuclei, and great loss in gross size.

If castration occurs within the first week of life, the seminal vesicles continue to develop to a limited degree. The side branches grow out from the central lumen, but the histological structure always remains simple, and no differentiation of the cells ever takes place. The gland grows slowly to about 30 days of age but does not usually exceed the size of that of a 15-day-old normal.

In the present experiments seminal vesicle tissue was transplanted from the same donors to the same respective hosts as were the prostate transplants in some of the earlier work. The results are given in Table 2. Although the number of grafts recovered is too small in many categories to give definite results, certain conclusions can be drawn as to the reactions of seminal vesicles when transplanted.

As with the prostate tissue, most of the young seminal vesicle transplants were between 5 and 12 days of age at the time of transplantation, with the great majority between 9 and 12 days of age.

It was difficult to classify clearly the seminal vesicle grafts, but the following categories were chosen: normal, with secretion granules, which meant that, in addition, the epithelium was high and the gland structure complex; castrate, when the graft showed the typical adult castration changes such as low epithelium and small nuclei; simple, when the lumen of the gland had few or no side branches; complex, when the side branches were ramified; degenerate; and with no glandular tissue. No further division of the simple and complex classifications was attempted on the basis of the state of the epithelium in such grafts. In some cases it was pseudostratified and with the cells crowded together; in others flat, squamous, and somewhat abnormal; and in still others definitely abnormal. No one type of epithelium was characteristic of tissue recovered from any one type of host. Host reactions were evident in the graft tissue in the form of lymphocytes and connective tissue infiltration, and these factors made it somewhat more difficult to interpret seminal vesicle reactions, particularly in regard to possible female hormone effects on fibrous and muscular tissue and epithelium.

A. TRANSPLANTS OF SEMINAL VESICLE TISSUE UNDER 35 DAYS OF AGE AT TRANSPLANTATION

1. *Into young and old normal males.*—Transplants of young seminal vesicles into young male hosts killed under 35 days of age (see Table 2) never had secretion granules, even though in several cases the graft tissue was over 40 days old. In grafts from young male hosts killed over 35 days of age, secretion granules were present in 4 grafts out of 6. The remaining 2, recovered from a host 38 days old, had a very complicated structure, but the grafts were evidently not receiving enough male hormone to elicit a completely positive response. In 2 out of the 4 positive grafts the graft tissue was only 33 days old, so the granules were formed somewhat precociously.

The weights of the grafts varied greatly, but the greatest androgenic stimulation, as judged by a weight of 125 mg., was found in a graft that had been transplanted for 155 days. The histological structure showed strong stimulation in the great distention of the lumen. Grafts that had been in normal young hosts for only 20–30 days usually weighed between 2 and 5 mg.

The seminal vesicle transplants in older males resulted in 6 out of 7 grafts with complicated structure and secretion granules. Distention in the lumina of the grafts frequently caused a stretching of the epithelium so that it was low and sometimes abnormal, but this seemed, as in the prostate grafts, the result of accumulation of secretion. In older male hosts there was evidence of far greater amounts of male hormone available for the grafts than in the young males, for positive grafts that had been in the host only 20 days weighed from 17 to 35 mg. and one with a graft period of 197 days weighed 159 mg.

2. *Into all other hosts, young and old.*—In the remainder of the grafts recovered from young hosts of all other classes, the histological structure of the grafts varied from a simple, open lumen to a moderately complex one, but no stimulation of the epithelium was ever found. This is in direct contrast to the findings in prostate grafts. No estrogenic effects on epithelium, fibrous tissue, or muscle were observed in the 6 grafts recovered from normal females, with 1 possible but questionable exception in which there seemed some hypertrophy of muscle.

In grafts from older hosts the tissue again varied from simple to complex, but evidence of androgenic stimulation was found in 2 grafts recovered from a female that had carried the grafts for 147 days and littered 6 times in that period. One of the grafts had very complicated structure, fairly high epithelium, and numerous granules, some with halos. The lumina were filled with secretion. The second graft was also very complicated and had a large amount of secretion in the lumen, but the epithelium was not so high and the granules not distinct. Both grafts gave the appearance of gland tissue that had been secreting. The graft weights were 28 mg. and 17 mg. A ventral prostate graft from the same host had distended acini and some high epithelium but no light areas and seemed in an early stage of regression.

It was difficult to recognize definite estrogenic effects because, as has been discussed, increases in connective tissue and abnormal epithelium were factors met, to some degree, in all classes of hosts, although they did not seem so evident in grafts from normal males. In some of the grafts from old females and from those bred repeatedly there were possible indications of muscle hypertrophy, but it was never striking. The weights of the recovered grafts, however, suggest that there may have been estrogenic influence, for the grafts from old females weighed, in over one-third of the cases, between 10 and 25 mg., while those from spayed females and castrated males weighed between 1 and 6 mg. This was definitely not a functional stimulation such as was found in grafts from male hosts, with the exception of the 2 cases noted above from a pregnant female. The increased weights of the grafts from females seemed to be caused by increase in connective tissue and possibly by muscle hypertrophy.

B. TRANSPLANTS OF SEMINAL VESICLE TISSUE OVER 35 DAYS OF AGE AT TRANSPLANTATION

In the experiments in which older seminal vesicle tissue was transplanted (see lower half of Table 2), only 27 per cent of the grafts were recovered, and almost one-half of these were degenerate or had no gland tissue. The numbers are so low that it is impossible to draw any conclusions except that adult seminal vesicle tissue was maintained in a functional state with large secretion granules in old male hosts for as long as 7 months. Again, the grafts varied in weight, but functional grafts weighing 38 mg. were recovered. No graft from any other host weighed more than 4 mg.

DISCUSSION

The transplantation of young ventral prostate tissue into young and old castrated males and spayed females was designed to bring out, if possible, whether such tissue has the capacity to differentiate regardless of the age or sex of the host. The results show that young prostate tissue transplanted into young castrated males and spayed females develops distended acini and epithelial light areas in a certain percentage of cases. This differentiation in the grafts resembles and parallels that of the prostate gland of the young castrated male rat and is maintained until the host is about 35 days of age, when regression begins.

When young prostate transplants are put into hosts which are over 35 days old at the time of transplantation, the tissue survives and grows in some cases, but none of the grafts ever develop acini that are widely distended or have any evidence of epithelial light areas. These findings lead to the conclusion that young prostate tissue does not seem to be capable of "self-differentiation" but is dependent upon androgenic stimulation, which is provided in the young castrated male and spayed female rat and not in older castrated males and spayed females. The source of such androgenic stimulation is the next question.

Howard (1937, 1938) corroborated the findings of differentiation of the ventral prostate in young castrated rats as reported by Price (1936) and suggested that the source of andromimetic material that could be capable of stimulating the prostate might be the juvenile cortex of the rat adrenal, since it is present at the time the prostate of the young castrated rat is developing to a differentiated state and has lost its distinctive character at about 35–40 days of age, when the prostate regresses. In further work Howard (1939) has shown a close relationship between the histological condition of mouse seminal vesicles and prostate and the presence in the adrenal cortex of either a primary or a secondary X zone. In rats Howard found that castration did not result in hypertrophy of the X zone, nor did the seminal vesicles differentiate after castration at ages corresponding to those at which castration was done on mice.

Other research gives further support to the theory that the adrenal gland may be responsible for androgenic stimulation of the prostate. Davidson and Moon (1936) administered adrenocorticotropic hormone to young castrated male rats and caused hypertrophy of the adrenal cortex and gross and histological stimulation of the prostate. Burrill and Greene (1939*a*) castrated a group of young male rats, castrated and adrenalectomized others, and adrenalectomized a third group. The prostates of the castrated rats and of the adrenalectomized rats were found at autopsy to be differentiated and contained epithelial light areas, but those of the rats that were both castrated and adrenalectomized were castrate in histological appearance. Gersh and Grollman (1939) performed somewhat similar experiments, with conflicting results. They used rats and mice that were castrated or castrated-adrenalectomized. All the latter animals were maintained by the oral administration of a cortical hormone which had been tested on castrated mice and had shown no androgenic activity. The size of tubules and cells in two lobes of the prostate and in Cowper's gland was measured, and the relative volume of the glands was computed. They found no difference between the glands of castrated and castrated-adrenalectomized animals. Their conclusion was that the adrenal cortex, more particularly the "juvenile" cells of rats and the X zone of mice, has no androgenic function.

Still other lines of evidence point to the adrenal cortex as a source of androgenic substance. Reichstein (1936, 1938), Hodler (1936, 1937), and Parkes (1937) found such a substance in adrenal extracts. Gersh and Grollman (1939) failed to demonstrate any such activity in extracts of adrenals of fetal pigs, fetal and newborn humans, and in extracts of X-zone-bearing adrenals of mice. In various clinical pathologies with adrenal involvement large amounts of androgenic substance have been recovered from the urine.

Although there is some disagreement as to the importance of the adrenal as a source of androgens, the evidence suggests that under certain conditions the adrenal cortex is capable of elaborating such substances in effective amounts and that the prostate of the rat and the prostate and seminal vesicles of the mouse respond, as evidenced by their behavior in the castrated animal. The seminal vesicles of the rat have been shown to have a much higher male hormone threshold than the rat ventral prostate, and this may account for their failure to differentiate in the presence of possible adrenal androgens.

The question of the amount of adrenal androgens circulating normally in rats is an interesting one. Regression of the prostate and seminal vesicles of adult rats follows within a few days after castration. This indicates (1) that the adult prostate tissue is less reactive or (2) that less extra-gonadal androgen is present in adults or (3) that both conditions obtain. It is possible that adult prostate requires more male hormone to maintain its normal histological state than young prostate tissue. However, the results of transplanting young prostates into old castrated males and spayed females show that there is definitely a smaller amount of extra-gonadal androgen in adult castrates and spays, for the transplants fail to differentiate although the tissue incorporates and grows. Howard (1939) has shown that castration in the adult rat does not result in differentiation of the adrenal cortex.

It must be assumed that in the young rat, both male and female, there are normally present androgens of adrenal origin, unless it is possible that removal of the gonads brings into action or increases a potential and temporary capacity. There are several facts that tend to disprove this latter suggestion and to indicate that some young normal females have adrenal androgens in effective amounts. Transplants of male ventral prostate tissue into young normal females have been shown in this work to differentiate and develop light areas, as do transplants in spayed females; and in both types of host the transplants are castrate in appearance when the hosts are over 35 days old at autopsy. In normal female hosts this is only a temporary condition, and older female hosts again yield positive grafts. A study of the normal development of spontaneously occurring female prostates (Price, 1939) brings out that in normal females in which such glands occur the female prostate develops light areas resembling those of the male prostate and maintains them until about 40 days of age, when the gland becomes castrate. The female prostates are highly variable in the degree of development they attain, and in this respect and in time of regression resemble the male prostate of the young castrated male. It was suggested (Price, 1939) that the development and regression of the prostate of normal females might depend upon the adrenal cortex. In work to be published elsewhere, it has been shown that female prostates can develop and differentiate in the absence of the ovaries. This has been reported recently by Burrill and Greene (1939*b*).

There is, however, a possibility that young ovaries produce small amounts of androgenic substance that may reinforce the action of adrenal androgens, so that the development of the female prostate in the young female may normally be influenced by both. The present findings in prostate transplants from young normal females and young spayed females give some support to this. The number of recovered grafts is too low to be more than suggestive; but, although grafts containing light areas were recovered from both types of hosts when autopsy was done under 35 days of age, there were indications in epithelial height, acinous diameters, and light areas that grafts from normal female hosts showed stronger stimulation. Further experiments are now in progress to test this point.

Burrill and Greene (1939*b*) report that transplants of male prostate put intraperitoneally in young normal females and spayed females that were killed at 20–35 days of age were always nonfunctional. Female prostates were found in 4 of the intact implanted females and showed evidence of functional activity. The spayed female hosts gave nonfunctional male grafts; but, out of 10 spayed females with female prostates, 4 of the prostates showed some evidence of function. From this they concluded that the threshold of reaction of female prostate tissue is below that of male prostate. In further experiments (Greene and Burrill, 1939*b*) they implanted young normal and spayed females with male ventral prostate tissue and injected the hosts with chorionic gonadotropic hormone. Positive implants were recovered from the normal female hosts but not from the spayed hosts, and the clitora of the normal females were enlarged. In hosts older than 28 days the implants were negative. Their conclusions were that the young, immature ovary can be stimulated to produce androgens in amounts sufficient to induce a functional state in male prostrate grafts but that in older females the ovary no longer can be stimulated.

The findings of Burrill and Greene (1939*b*) that male prostate grafts were nonfunctional in young normal and spayed female hosts between 20 and 35 days of age are not in agreement with the results reported here. It must be stressed that prostate tissue put into a foreign position in a host is not entirely comparable to the host prostate. Implants are subjected to a number of unfavorable influences, one of which is changed vascularity. In our work it was found that in almost all cases grafts showed a lower degree of androgenic stimulation as judged by a comparison of epithelial height and light areas and acinous diameters than the host prostate. In our experiments in male prostate transplants recovered from young normal females and spayed females there was variability in histological condition, but there was no question as to the functional state of some of the grafts.

Grafts recovered from female hosts older than 35 days but younger than 80 days were castrate in histological condition. This could be interpreted to mean, since that age range corresponds to a time when the adrenal androgens would be expected to disappear or be markedly reduced in amount, that the grafts reflected the lack of adrenal stimulation. The findings of Bradbury and Gaensbauer (1939) and Greene and Burrill (1939*b*) tend to suggest that the young ovary is limited in the period in which it can be stimulated to produce detectable amounts of androgen. The present transplantation experiments indicate that young ovaries may normally be producing a small amount of androgenic substance. If the ovary is limited in its androgenic capacity, the lack of functional activity in grafts in females in the period from approximately 35 days to nearly 80 days may possibly be due to a drop in androgens, both from the adrenals and from the ovaries.

Although the young ovary may or may not be normally producing effective amounts of androgens, there is no question that ovaries of adult rats do so. Male prostate grafts in old females (over 80 days of age) were developed and maintained in normal histological condition for months. This was true both for virgin female hosts and for those bred continuously. No differentiation was ever attained by grafts in old spayed females. It was suggested (Price, 1939) that the normal female prostate might have a higher hormonal threshold than the male prostate from the following facts. Male prostate grafts in old females are functional, as reported by Price (1937) and in the present paper. All normal female prostates found so far in old females have been in a somewhat castrate state, although not greatly regressed.[2] Price (unpublished) has found that male prostate grafts recovered from old females having normal female prostates show light areas, while the female prostate, in spite of its more favorable position, has a histological state below that of the graft. Burrill and Greene (1939*b*) have interpreted their results to mean that the female prostate has a lower hormonal threshold, because in their work male prostate grafts were always nonfunctional in young females, even though the host female prostate was functional. A series of experiments on threshold differences as studied by male and female prostate transplants is in progress and will be reported elsewhere.

Other workers have shown that ovaries can stimulate male accessory glands. Hill and Gardner (1936), Hill (1937*a* and *b*), Hill and Strong (1938), and Deanesly (1938) have shown that ovarian grafts put into the ears of castrated male mice and rats will maintain the reproductive accessories, prostate, and seminal vesicles at a normal size and in a normal histological condition. Hill attributes the androgenic activity to the lowered temperature of the ear, which he assumes brings out a potential capacity of the female gonad. Deanesly correlates androgenic activity of the ovary with thecal luteinization of the ovarian graft.

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[2] While this paper was in press, functional female prostate glands with cell light areas present were found in two normal adult female rats.

Ovaries have been shown by many workers to produce substances with androgenic action under a variety of conditions. Transplanted ovaries have maintained normal male accessory glands in castrated guinea pigs (Lipschütz, 1932) and mice (De Jongh and Korteweg, 1935). Removal of one ovary and partial removal of the other caused masculinization of female guinea pigs (Lipschütz, 1933, 1937). Steinach and Kun (1931) produced masculinization of female guinea pigs by irradiation with x-ray and with injection of gonadotropic substances. Guyénot and his collaborators have obtained masculinization of female guinea pigs by injection with extracts of hypophyses and with urine of pregnant women (Guyénot, Ponse, and Wietrzykowski, 1932; Guyénot, Ponse, Dottrens, Vallette, and Trolliet, 1933; Guyénot and Naville-Trolliet, 1936) and observed masculinization in female guinea pigs of an interspecific cross—*Cavia aperea* ×*C. cobaya* (Guyénot and Duszynska-Wietrzykowski, 1935). Papanicolaou and Falk (1934) also reported masculinization of female guinea pigs by injection of pregnancy urine extracts. Recently Greene and Burrill (1939*a* and *b*) and Bradbury and Gaensbauer (1939) have induced masculinization in young female rats with gonadotropic extracts.

All these researches, including the present report show that ovaries of some female mammals are capable of producing substances which stimulate male accessory gland and have an androgenic action on the clitoris of females. Luteinization or thecal luteinization of the ovaries has been thought by some workers to be associated with such androgenic action. It has been shown that extracts of corpora lutea, progesterone, and progestin are capable of stimulating male accessory glands in young castrated rats (Lamar, 1937; Greene, Burrill, and Ivy, 1939). The latter suggest that the androgenic action produced by transplanted ovaries and by those stimulated with A.P.L. may be due to progesterone. Hill and Strong (1938) and Deanesly (1938) conclude that transplanted ovaries produce an androgen that is not testosterone. The nature of the ovarian-produced androgen seems to be still an open question.

No estrogenic effects were observed in prostate grafts put into normal females. In seminal vesicle grafts there were indications in the histology and also in graft weights that host ovaries might be causing some increase in fibrous and muscular tissue. Pfeiffer (1936) transplanted ovaries into castrated male rats and reported hypertrophy of muscle in host seminal vesicles and coagulating glands. The epithelium of the coagulating gland became stratified, squamous, and, in extreme cases, cornified. Hyperplasia and metaplasia were found in the lateral lobe of the prostate.

Transplants of prostates and seminal vesicles have been used by several workers as indicators of male hormone presence or as a means of studying the reactions of these glands in the presence of introduced hormones. Fisher (1923) transplanted guinea pig and rat seminal vesicles and showed that seminal vesicle grafts would persist and appear functional in normal male hosts. Heckel and Kretschmer (1935) made autotransplants of rabbit prostate into the anterior chamber of the eye and obtained successful growth, which could be observed easily. Some increase in size of the transplants was noted after injection of antuitrin S, and hyperplasia and hypertrophy of the glandular structure occurred. R. A. Moore and collaborators (Moore, Rosenblum, Tolins, and Melchionna, 1937; Moore, Melchionna, Tolins, and Rosenblum, 1937; Moore and Smith, 1937) transplanted rabbit prostate and seminal vesicle into the anterior chamber of the eye and photographed the implants daily. They found a daily variation in size, which was markedly diminished or absent after castration. Slight or moderate temporary increase in size of the transplant in normal males was observed after injections of gonadotropic substance from pregnancy urine, alkaline extracts of anterior pituitary, male hormone, and female hormone. In castrated males all pituitary derivatives were ineffective, but male and female hormones caused increase in the size of the grafts. Corpus luteum hormone had no effect. A correlated elevation of blood cholesterol and increase in size of ocular transplants were found in male rabbits injected with female sex hormone. Ingestion of cholesterol had no effect on transplant size. In all this work no differences in reaction were found between the prostate and the seminal vesicle.

Melchionna and Flanders (1938) transplanted seminal vesicle tissue into the eyes of female rabbits and found that daily variations in size occurred in female hosts and were less conspicuous when the hosts were spayed. Injections of gonadotropic hormone caused slight increases in size, and both male and female hormones produced size increases in implants in normal and spayed hosts. Corpus luteum injections had no effect.

Turner (1938) used intraocular transplants of rat prostate and seminal vesicle as indicators of hormone production of intraocular testis transplants.

SUMMARY AND CONCLUSIONS

I. Experimental results
   A. Young ventral prostate transplants will grow and differentiate and possess a normal functional histological state:
      1. In young or old normal males for long periods.
      2. In young normal females until a host age of approximately 50–60 days.
      3. In young normal females killed at a host age of over 80 days or in old normal females (virgin or bred and lactating) for months.
      4. In young castrated males, or spayed females, to a host age of about 40 days.
   B. Young ventral prostate transplants will be incorporated and be maintained but will undergo little or no development beyond their stage at time of transplantation:
      1. In old castrated males.
      2. In old spayed females.
   C. Old ventral prostate tissue will be maintained in a normal functional histological condition:
      1. In old normal males for months.
      2. In young normal males for months.
   D. Young seminal vesicle transplants will grow and differentiate and possess a normal functional histological state:
      1. In young or old normal males for months.
      2. Possibly in some females that have been bred repeatedly for long periods.
   E. Old seminal vesicle transplants will grow and differentiate and possess a normal functional histological state:
      1. In old normal males for months.

II. Conclusions
   A. Ventral prostate tissue, which is normally under control of male hormone from testes, is stimulated by androgens from the adrenals in young castrated males,

young normal females, and young spayed females, and by ovarian androgens in older females, and possibly to some degree in young females.

B. Seminal vesicle tissue presumably has too high a hormone threshold to react to these influences except for an indication of androgenic stimulation in grafts in old females bred repeatedly, in which case ovarian androgens were probably effective.

LITERATURE CITED

Bradbury, J. T., and Gaensbauer, F. 1939. Proc Soc. Exper. Biol. and Med , **41**:128.
Burrill, M. W., and Greene, R. R. 1939*a*. Proc. Soc. Exper. Biol. and Med., **40**:327.
———. 1939*b*. *Ibid.*, **42**:764.
Davidson, C. S., and Moon, H. D. 1936. Proc. Soc. Exper. Biol. and Med., **35**:281.
Deanesly, R. 1938. Proc. Roy. Soc. London, B, **126**:122.
Fisher, N. F. 1923. Amer. Jour. Physiol., **64**:244.
Gersh, I., and Grollman, A. 1939. Amer. Jour. Physiol., **126**:368.
Greene, R. R., and Burrill, M. W. 1939*a*. Proc. Soc. Exper. Biol. and Med., **40**:514.
———. 1939*b*. *Ibid.*, **42**:761.
Greene, R. R.; Burrill, M. W.; and Ivy, A. C. 1939. Endocrinology, **24**:351.
Guyénot, E., and Duszynska-Wietrzykowska, Mme. 1935. Rev. suisse zool., **42**:341.
Guyénot, E., and Naville-Trolliet, Mme I. 1936. Rev. suisse zool., **43**:415.
Guyénot, E.; Ponse, K.; Dottrens, E.; Vallette, M.; and Trolliet, I. 1933. Rev. suisse zool., **40**:217.
Guyénot, E.; Ponse, K.; and Wietrzykowska, J. 1932. Compt. rend. Acad. sci , **194**:1051.
Heckel, N. J., and Kretschmer, H. 1935. Surg., Gyn. and Obst., **61**:1.
Hill, R. T. 1937*a*. Endocrinology, **21**:495.
———. 1937*b*. *Ibid.*, p. 633.
Hill, R. T., and Gardner, W. U. 1936. Anat. Rec., Suppl., **64**:21.
Hill, R. T., and Strong, M. T. 1938. Endocrinology, **22**:663.
Hodler, D. 1936. Compt. rend. Soc de biol., **122**:512.
———. 1937. Arch. d'anat., d'histol., et d'embryol., **24**:1.
Howard, E. 1937. Amer. Jour. Physiol., **119**:339.
———. 1938. Amer. Jour. Anat., **62**:351.
———. 1939. *Ibid.*, **65**:105.
Jongh, S. E. de, and Korteweg, R. 1935. Acta brev. neerl., **5**:126.
Lamar, J. K. 1937. Anat. Rec., Suppl., **70**:45.
Lipschütz, A. 1932. Virchow's Arch. f. Path. Anat , **285**:35.
———. 1933. Compt. rend. Soc. de biol., **112**:1272.
———. 1937. Nature, **140**:892.
Melchionna, R. H., and Flanders, S. 1938. Endocrinology, **23**:468.
Moore, R. A.; Melchionna, R. H.; Tolins, S. H.; and Rosenblum, H. B. 1937. Jour. Exper. Med., **66**:281.
Moore, R. A.; Rosenblum, H. B.; Tolins, S. H ; and Melchionna, R. H. 1937. Jour. Exper. Med., **66**:273.
Moore, R. A., and Smith, J. J. 1937. Jour. Exper. Med. **66**:291.
Papanicolaou, G., and Falk, E. A. 1934. Proc. Soc. Exper. Biol. and Med., **31**:750.
Parkes, A. S. 1937. Nature, **139**:964.
Pfeiffer, C. A. 1936. Anat. Rec., **65**:215.
Price, D. 1936. Amer. Jour. Anat., **60**:79.
———. 1937. Anat. Rec., Suppl., **70**:60.
———. 1939. Proc. Soc. Exper. Biol. and Med., **41**:580.
Reichstein, T. 1936. Helvet. chim. acta, **19**:223.
———. 1938. Ergebn. d. Vitamin- Hormon-Forsch., **1**:334.
Steinach, E., and Kun, H. 1931. Arch. f. d. ges. Physiol., **225**:266.
Turner, C. D. 1938. Amer. Jour. Anat., **63**:101.
Wiesner, B. P. 1934. Jour. Obst. and Gyn. Brit. Emp., **41**:867 and **42**:8.

PLATE I

FIG. 1.—Ventral prostate graft 79 days of age. Transplanted at 11 days of age into a 68-day-old female and allowed to remain 68 days. Host 126 days old at autopsy and had littered twice during the graft period and was pregnant again at autopsy. Note the distention of the acini and their normal relationship. Light areas in the cells are apparent. ×250. Graft weight, 52 mg.

FIG. 2.—Ventral prostate graft 57 days of age. Transplanted at 15 days of age into a 141-day-old virgin female and allowed to remain for 42 days. Host 183 days old at autopsy and had remained unbred. Note the somewhat abnormal relationship of the acini and their small size, but the presence of high epithelium and strong cell light areas. ×250. Graft weight, 3 mg.

FIG. 3.—Ventral prostate of 25-day-old castrated male, host for graft in Figure 4. Castrated at 5 days of age. ×250.

FIG. 4.—Ventral prostate graft 30 days of age. Transplanted at 10 days into a 5-day-old castrated male and allowed to remain for 20 days. Host 25 days old at autopsy. Note similar acinous distention and cell light areas in graft and in host prostate in Figure 3. ×250. Graft weight, 5 mg.

PLATE II

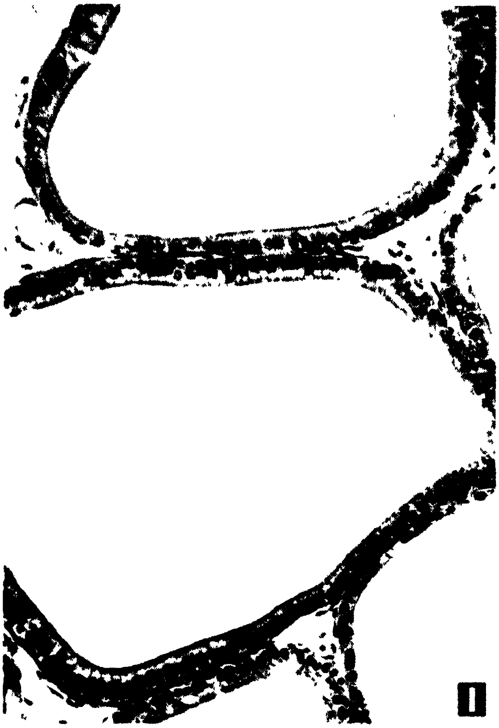
FIG. 5.—Ventral prostate of 56-day-old castrated male, host for graft in Figure 6. Castrated at 11 days of age. ×250.

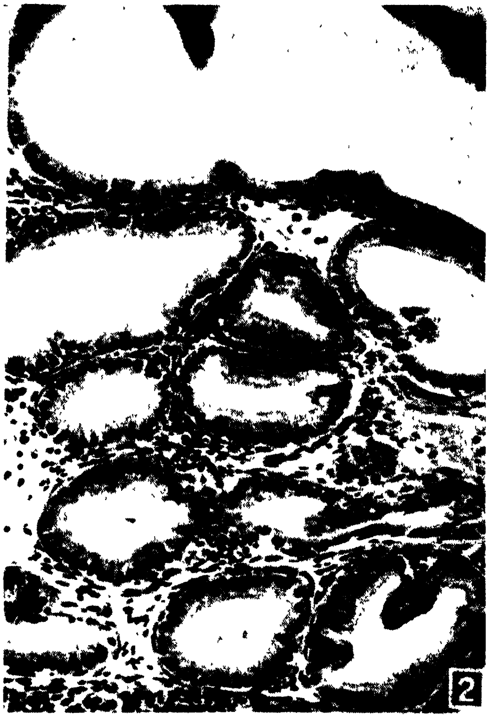
FIG. 6.—Ventral prostate graft (autotransplant) 56 days of age. Transplanted at 11 days and allowed to remain for 45 days. Host 56 days old at autopsy. Note castrate condition of host prostate (Fig. 5) and prostate graft. ×250. Graft weight, 2.1 mg.

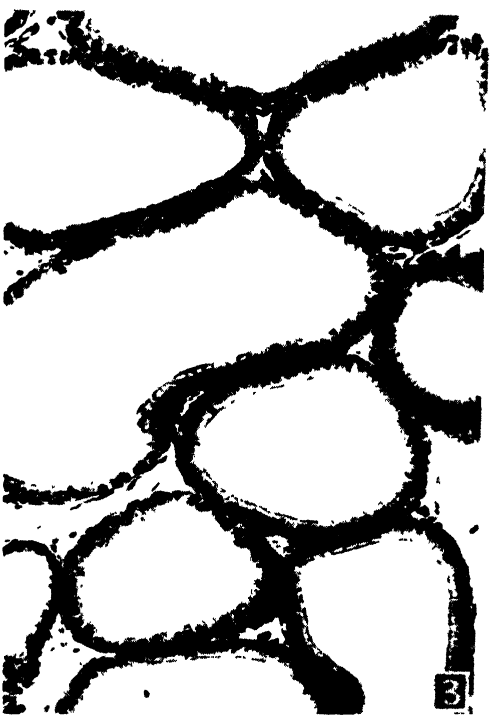
FIG. 7.—Ventral prostate graft 28 days of age. Transplanted at 10 days into a 47-day-old male (castrated at the time of implantation) and allowed to remain for 18 days. Host 65 days old at autopsy. Note the small acini and small dark nuclei and compare with Figure 8. ×250. Graft weight, 2 mg.

FIG. 8.—Ventral prostate 11 days of age. Tissue typical of young ventral prostates at the time of transplantation. Note the presence of some high epithelium and pale light areas. ×250.

PLATE I







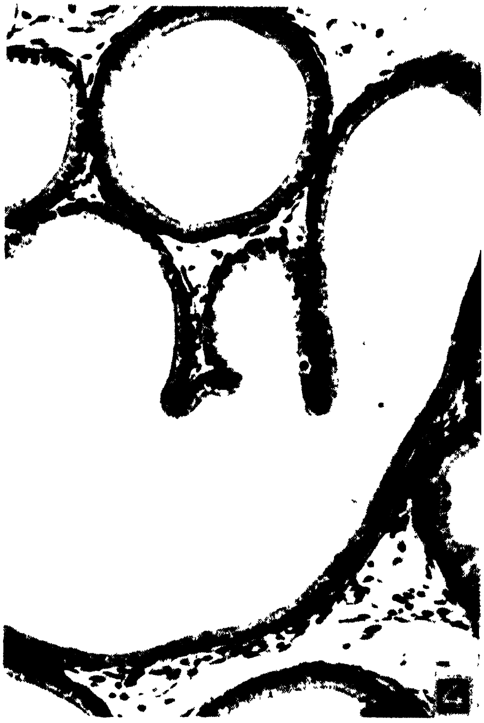
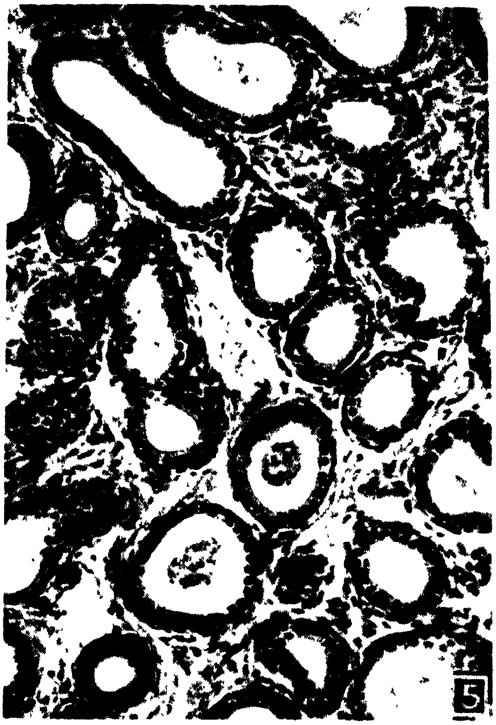


PLATE II



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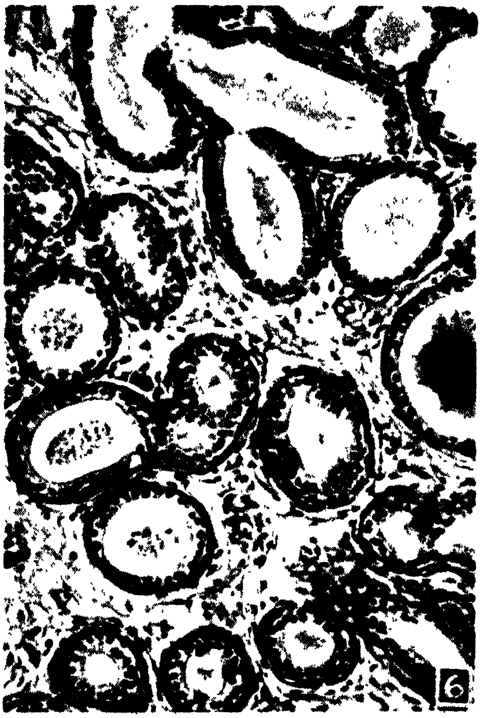

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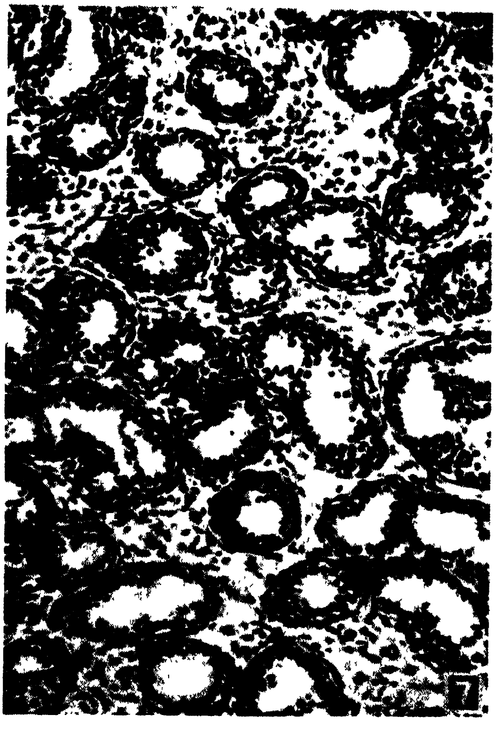

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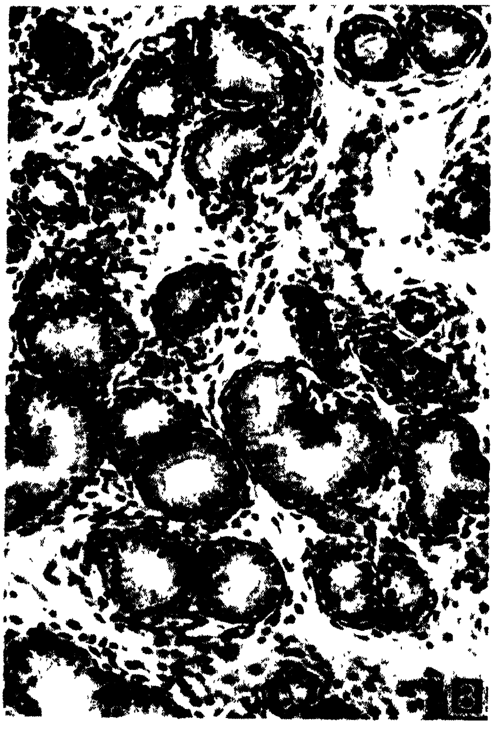

8

SEQUENCE OF ROENTGEN RAY EFFECTS ON THE DEVELOPMENT OF THE GRASSHOPPER EGG. I. EFFECTS ON SUBSEQUENT DEVELOPMENT OF IRRADIATING 1-DAY EGGS[1]

(Seven figures)

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MOST of the investigations on biological effects of roentgen radiation have been restricted to a single criterion. In many cases the effect studied has been cytological injury, in some it has been retardation of growth, and in others the percentage of individuals killed. Few have attempted the correlation of these cytological and physiological effects. Such a correlation is of interest in determining which changes after irradiation are primary and which are secondary in nature. The grasshopper (*Melanoplus differentialis*) egg is a favorable material for such a study because it is well known both from the morphological and from the physiological standpoints.

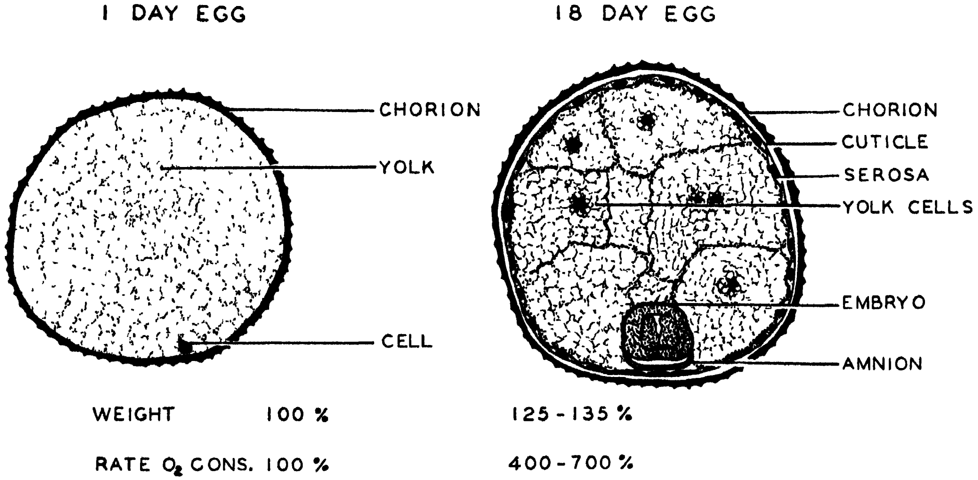

FIG. 1.—Diagrammatic cross-sections to show some of the changes which take place in 18 days at 25° C.

Several changes occur during the development of the grasshopper egg which respond to x-radiation, and these may be used as measurements of radiation injury. Figure 1 shows, diagrammatically, some changes which occur during the first 18 days of development at 25° C. These changes which can be studied quantitatively, are (1) growth of the embryo, (2) production of yolk and serosa cells, (3) increase in rate of oxygen consumption, and (4) increase in gross weight. It is possible, by using these as criteria, to employ several methods of studying radiation injury on the same organism. The relative radiosensitivity of the various processes undergoes marked change at several stages in the development of the egg (Evans, 1934, 1935, 1936*a* and *b*, 1937, 1938). For this reason the investigation has been divided into several parts, each dealing with a particular stage in development. This report is the first of the series and deals with effects (on subsequent development) of irradiating the eggs on the first day after laying. The effects of radiation on 1-day eggs are in marked contrast to those which occur in eggs rayed at other stages of development.

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[1] Aided by grants from the Committee on Radiations of the National Research Council, Rockefeller Foundation Fund for Research on Physiology of the Normal Cell, and the Committee on Grants of the American Association for the Advancement of Science.

[2] It is a pleasure to acknowledge the assistance of Professors J. H. Bodine and H. D. Kerr, whose interest and help have made this investigation possible. Thanks are due also to Dr. E. H. Slifer for her constructive criticism and for the loan of the slides of whole eggs, which were used in preparing the diagrams of Fig. 1.

MATERIAL AND METHODS

The radiation (130 kv., 5 ma., cardboard filter only, target distance, 30 cm.) was applied in one treatment, and the amount was increased by lengthening the exposure time.

The sand in the grasshopper cages was cleared of eggs at 10:00 P.M., and the eggs were collected at 8:00 A.M. the next day. Unless otherwise specified, the eggs used in this investigation were irradiated in the afternoon of the first day so that they ranged in ages from 12 to 24 hours when exposed to the radiation. Eggs from several pods were used so that each lot consisted of from 100 to 300 eggs at the beginning of the experiment. Eggs were taken from each lot at intervals of 2–3 days, and the embryos examined. The reader is referred to the paper of Slifer (1932) for the morphological changes during development at 25° C. All these experiments were performed at this temperature, and moisture was controlled by keeping the eggs on moist sand.

EFFECTS OF THE RADIATION ON DEVELOPMENT AND RATE OF OXYGEN CONSUMPTION

The most striking result of irradiating 1-day eggs was the all-or-none character of the injury to the embryos. The irradiated eggs examined at the end of the experiment (30 days) contained either a whole embryo or none at all. This is in marked contrast to the results of irradiating older eggs, where partial injury was obtained. This change in the character of the injury has been reported in more detail in a previous paper (Evans, 1937). In the present investigation it was found that dosages of 100 and 200 r prevented the formation of embryos in some eggs but that the development of the surviving embryos was not visibly affected except that they appeared to be growing more slowly than the controls. The effect of retarding the development of the embryo was more evident following dosages of 400 and 500 r.

The rate of morphological development was closely paralleled by the increase in oxygen-consumption rate. The measurements of oxygen consumption were made at 25° C. in small Krogh respirometers of the type developed in this laboratory. Determinations were made on lots of 20 eggs, and the same manometer was used for each experimental lot and its control throughout the experiment. The curve of oxygen consumption for the controls during the experiment is shown in Figure 2 (Curve 1). It will be observed that the rate of oxygen consumption increases very rapidly after the fourth day and reaches a maximum about the eighteenth. The rate then decreases to a minimal level and remains low for an indeterminant period (diapause).

The retardation of developmental rate and oxygen consumption, as a result of irradiation with moderate dosages, is shown in Curves 2 and 3. The lower peak of the experimental curves is probably related to the fact that many of the irradiated eggs contained no embryos. It will also be observed that the oxygen-consumption rates (Curves 2 and 3) did not drop to the level of the controls at diapause by the twenty-seventh day. This was probably due to the fact that the embryos had not reached the morphological stage at which the controls go into diapause and were, therefore, still developmentally active. Later the embryos ceased growing, and the oxygen-consumption rate dropped to the minimal level. This phenomenon of diapause has been studied extensively by Bodine (1923, 1929, 1932*a*, and 1932*b*).

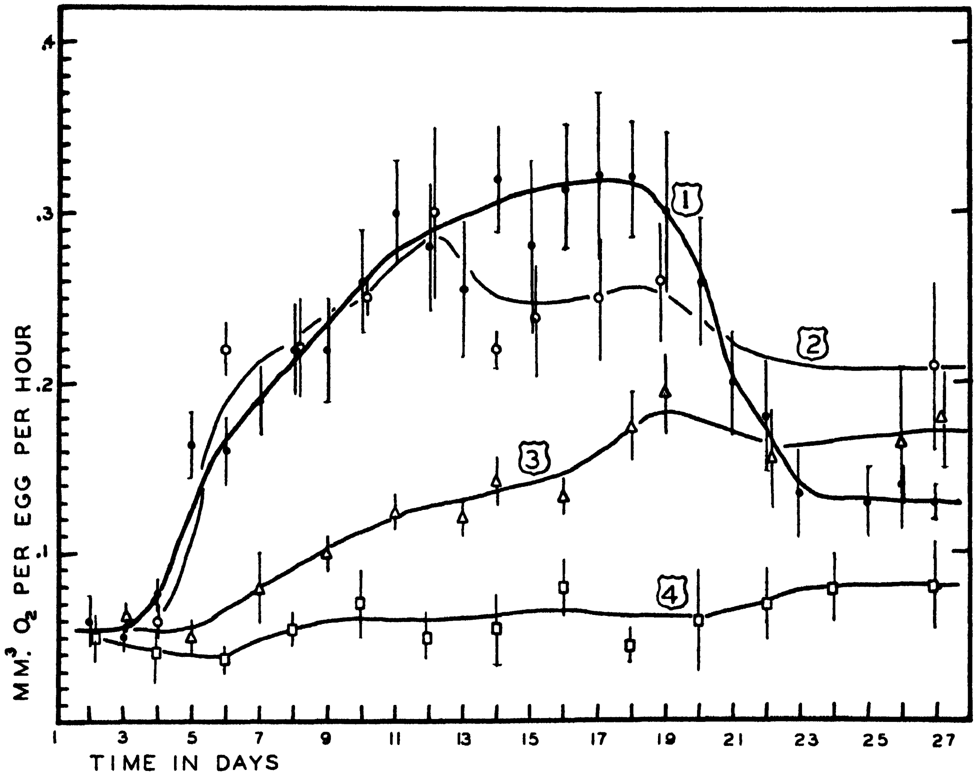

FIG. 2.—Effects of roentgen radiation on oxygen consumption and development. The means and standard errors of the means of 17 lots of controls shown in Curve 1. Curve 2 is of means and standard errors of 4 lots of eggs rayed 100–300 r. The means and standard errors of eggs rayed 400, 500, and 600 r are shown in Curve 3. Curve 4 is of lots rayed 800, 1,000, 2,500, and 3,200 r and is drawn through the mean.

In these experiments dosages of over 800 r completely inhibited embryo formation in all eggs. The oxygen-consumption rate of such eggs (Curve 4) remained at about the initial level. It appears, therefore, that the increase in oxygen consumption during prediapause is dependent on the formation and activity of the embryo cells.

EFFECT ON INCREASE IN GROSS WEIGHT

It was observed that during the prediapause development the controls and some of the irradiated eggs gained in gross weight. This gain in weight is evidently due to the fact that eggs take up water. Slifer (1937, 1938) has recently reported observations and experiments on the development of the egg membranes and the water-absorbing area.

Some eggs were weighed at the end of the experiments (early diapause), and the results are shown in Figure 3. It was found that dosages below 800 r reduced the mean gain in weight and dosages above this amount prevented any gain at all. This finding indicates that complete inhibition of development prevents the increase in gross weight that one finds occurring in the prediapause development of the unirradiated eggs.

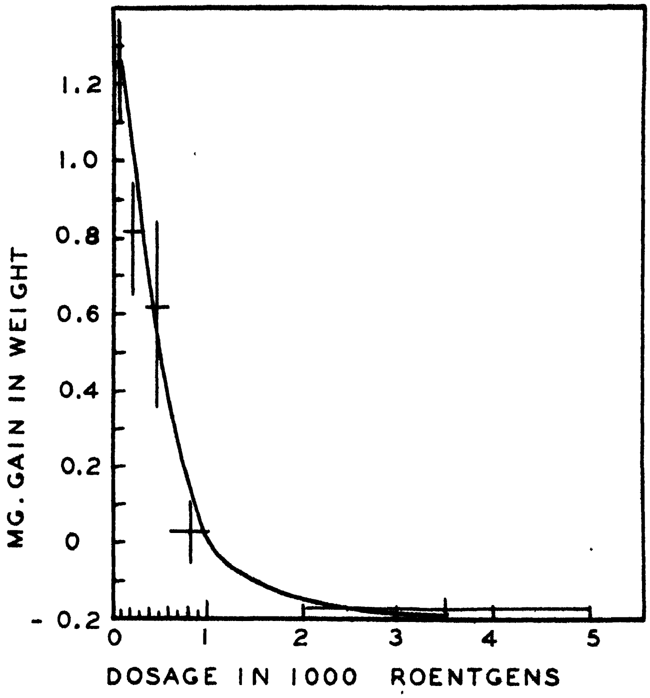

FIG. 3.—Means and standard errors of weights of controls and of 4 groups of irradiated eggs, at the end of the experiment, expressed as milligrams gain over that of the day irradiated. The initial weight was taken as 3.4 mg., which was the mean weight, on the first day, of 13 lots. The controls consisted of 19 eggs, and they all contained embryos. The 100–300 r group consisted of 14 eggs, and the 2 of this group which did not contain embryos had not gained in weight. Eight eggs were in the lots rayed with dosages of 400–500 r, and only 1 did not contain an embryo. The lots of 600–1,600 r consisted of 7, and none of the eggs contained an embryo. Those irradiated with dosages of 2,000–5,000 r were 8 in number, and none of these contained an embryo.

The relation between the increase in gross weight and the development of the embryo can be seen also by following the weights of control and irradiated eggs during the prediapause period. Curve 1 of Figure 4 shows that the weight increases rapidly after the embryo is formed (fifth to sixth days) and reaches the maximum at diapause. In an experiment in which eggs had been irradiated with a dosage of 500 r it became apparent by the tenth day that the eggs varied in size. From that time until the end of the experiment the eggs were selected for size, weighed individually, and then dissected. The weights of those eggs found to contain embryos are indicated in Curve 2 (solid squares). Curve 3 (open squares) is drawn through the weights of eggs which did not contain an embryo. It can be seen from an inspection of the graph that the weights of the eggs containing embryos were higher than those of eggs in which the embryo was missing.

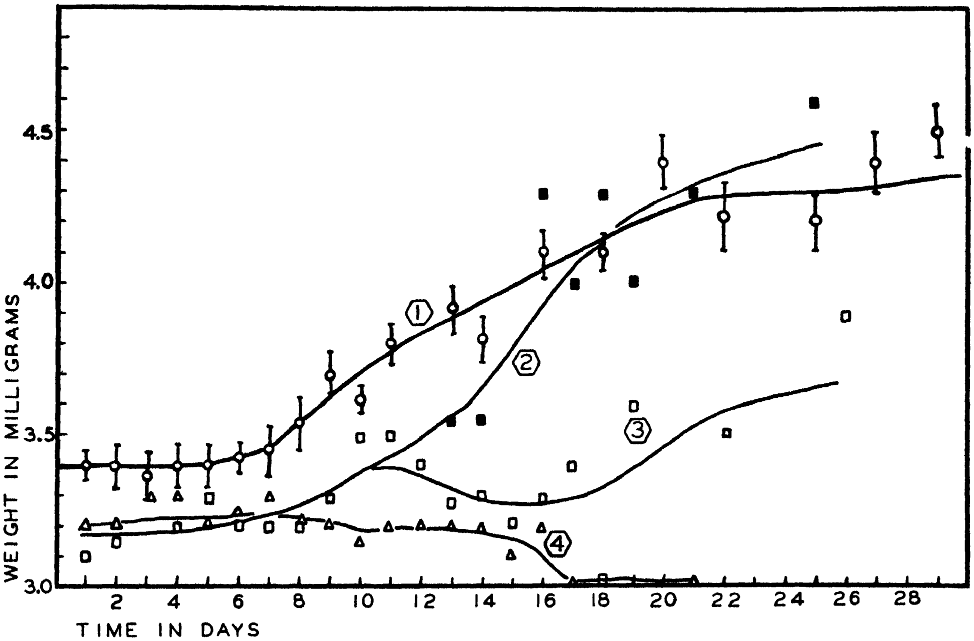

FIG. 4.—Curves showing effects of roentgen radiation on the subsequent weights of eggs during the prediapause period of development. Curve 1 shows the means and standard errors of 13 lots of control eggs. Curves 2 and 3 are of a lot of eggs rayed with a dosage of 500 r. From the tenth day the curves are distinguished on the basis of presence or absence of an embryo. Curve 2 (drawn through solid squares by inspection) is of eggs found to contain embryos. Curve 3 (through open squares) is of eggs in which embryo formation had been prevented. Curve 4 is of a lot of eggs irradiated with a dosage of 2,500 r. No embryos were found in any of these eggs.

One lot of eggs was irradiated with a dosage of 2,500 r and weighed daily for 21 days. The results are shown in Curve 4. The eggs failed to gain, and after 16 days they actually had decreased in weight. It is, therefore, concluded that, as in the case of oxygen-consumption rates, the increase usually seen in prediapause development is related to the development of the living constituents of the egg rather than to chemical or physical changes alone.

EFFECTS ON YOLK AND SEROSA NUCLEI

The curves of Figure 5 are survival curves in that they represent the number of yolk and serosa nuclei per egg when examined at the end of the experiment. The top curve, *A*, represents eggs between 2 and 12 hours of age when irradiated; the lower curve, *B*, eggs between 12 and 24 hours of age when treated. The eggs were examined when the controls were in early diapause. The number of yolk and serosa nuclei was determined by dissecting the egg in a drop of 5 per cent acetic acid, staining with Janus green, mounting in glycerine, and counting all the nuclei of the entire slide. Nuclei were counted instead of cells, as some of the cells contained more than 1 nucleus. The advantage of this kind of a preparation is in the ease and rapidity of the preparation with minimal loss of nuclei. These slides can be studied within 10 or 15 minutes and last very well for several days. In order to check the reliability of this procedure, one slide was counted 10 times and a mean value of 1,225 was obtained. The standard deviation of the mean was 77, and the standard error of the mean was 24.5, or 4 per cent. The smallest difference between irradiated and control eggs was about 25 per cent, so it appeared that this method of counting the nuclei was sufficiently accurate. The mean for the 55 control eggs was 873, with a standard error of the mean of 19.7. The results shown in Figure 5 indicate that the number of surviving yolk and serosa nuclei was reduced by the radiation. The survival data of the older eggs, which were plotted on semilogarithmic paper, fluctuated around a straight line, thus indicating that the dose-effect curve approximated an exponential one. The survival data for the younger eggs show too great an individual variation to place any interpretation on the character of the curve. The two curves are not significantly different, as the greater survival at 800 r, 1,600 r, and 2,000 r of the *A* curve over that of the *B* group was found to be only 0.265, 0.33, and 0.48 times the standard error of the difference. In the eggs rayed 2–12 hours after laying, the dosages of 1,600 r, 2,000 r, and 2,400 r resulted in reduction of survival numbers which were 1.48,

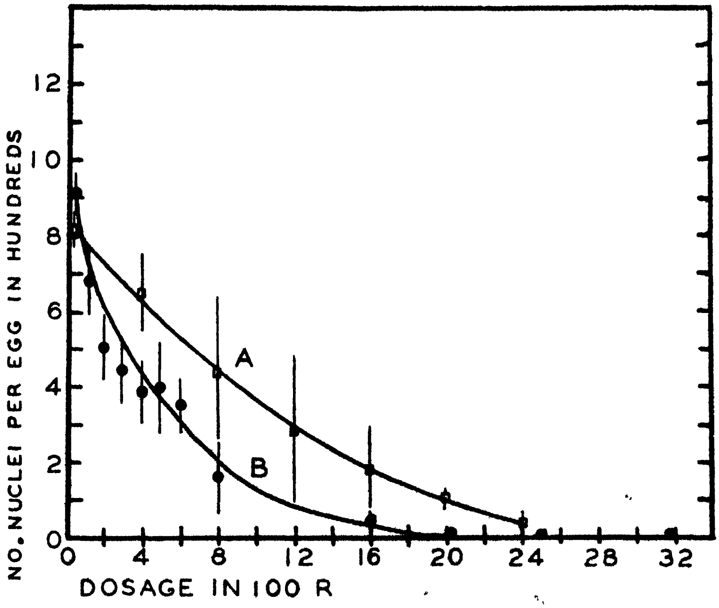

FIG. 5.—A graph showing the effect of roentgen radiation on decrease in the number of yolk and serosa nuclei surviving at diapause. The upper curve, *A*, is of eggs rayed when between 2 and 12 hours of age. The lower curve, *B*, is of eggs between 12 and 24 hours of age when irradiated.

2.4, and 3.4 times the standard error. Only the dosages of 2,000 r and 2,400 r reduced the number of surviving nuclei to a statistically significant degree. In the older eggs—12–24 hours of age—a dosage of 800 r produced a decrease which was 2.34 times the standard error. The decrease for the dosages of 1,600 r, 2,000 r, and 2,500 r were even more significant (2.6, 2.95, and 2.95 times the standard error).

An examination of Figure 6 will reveal three characteristics of the response of 1-day eggs to irradiation. In the first place, it is evident that the resistance of embryo formation varied in a marked degree. Some of the eggs irradiated with dosages as low as 200–400 r failed to form an embryo, whereas a few eggs were found to contain embryos even when irradiated with a dosage of over 1,000 r. Another characteristic of the effects on these eggs is the close relationship between presence of embryo and presence of serosa and yolk nuclei. It appears, especially in Group A, that the radiation either prevented formation of both embryo and yolk-serosa cells or allowed both to develop. In the *B* group there is some indication that the yolk and serosa cells were slightly more resistant than the embryos. A third observation to be made of the data in Figure 6 is that increasingly heavy dosages decreased the number of eggs containing the full complement of nuclei and increased the number which contained none at all rather than decreasing the number per egg. This is more evident in Group A than in Group B.

Five eggs of Group A were examined at the time they were irradiated, and the mean number of nuclei found was 1.6 ± 0.54. Several eggs have been examined between the twelfth and twenty-fourth hour, and the mean of this group was 9 ± 1.2 nuclei per egg. Slifer and King (1934) have sectioned *M. differentialis* eggs and have found eggs 9 hours after laying to have from 1 to 3 nuclei present. Eggs 1 day of age were found to have from 61 to 32 segmentation nuclei present. It was found in the present investigation that during the second day of development the number of cleavage nuclei increased rapidly. An egg examined at 10:00 A.M. on the second day contained 10 nuclei, at 2:00 P.M. another egg contained 36, and one egg examined at 5:30 P.M. was found to have 134 nuclei. During the third day, the number continued to increase rapidly, and counts of 183 and 269 were made. On the fourth day an egg was found to have 455 nuclei, which were very small at this time. On the fifth day the number of nuclei reached a maximum of 1,033. Cells of the embryo could easily be distinguished, and many of the others appeared to be differentiating into serosa cells. The nuclei then increased in size, and typical serosa and yolk cells were found in the microscopic preparations. In another lot of control eggs the yolk and serosa cells could be distinguished by the fourth day. In all cases the cleavage cells appeared to go through a period of rapid mitotic activity for 2 or 3 days, during which time the cells decreased in size. This was followed in the next few days by continued mitotic activity of the cells in the germ band, but those of the yolk and serosa ceased division, became large, and differentiated into an appearance typical of those of the rest of the diapause period. This early development presents many interesting phenomena, but interpretations should await further investigation.

Effects of radiation on the cells can be detected as early as the day following the treatment. The effects noted were (1) abnormal mitoses, (2) pyknosis, (3) phagocytosis, and (4) a reduction in number of nuclei as compared to the controls.

For purposes of clarity this discussion is limited to the number of surviving yolk and serosa nuclei (completely formed), so the effect of radiation on lowering the cell number is not evident until the fifth day when the cells would normally have assumed the typical condition.

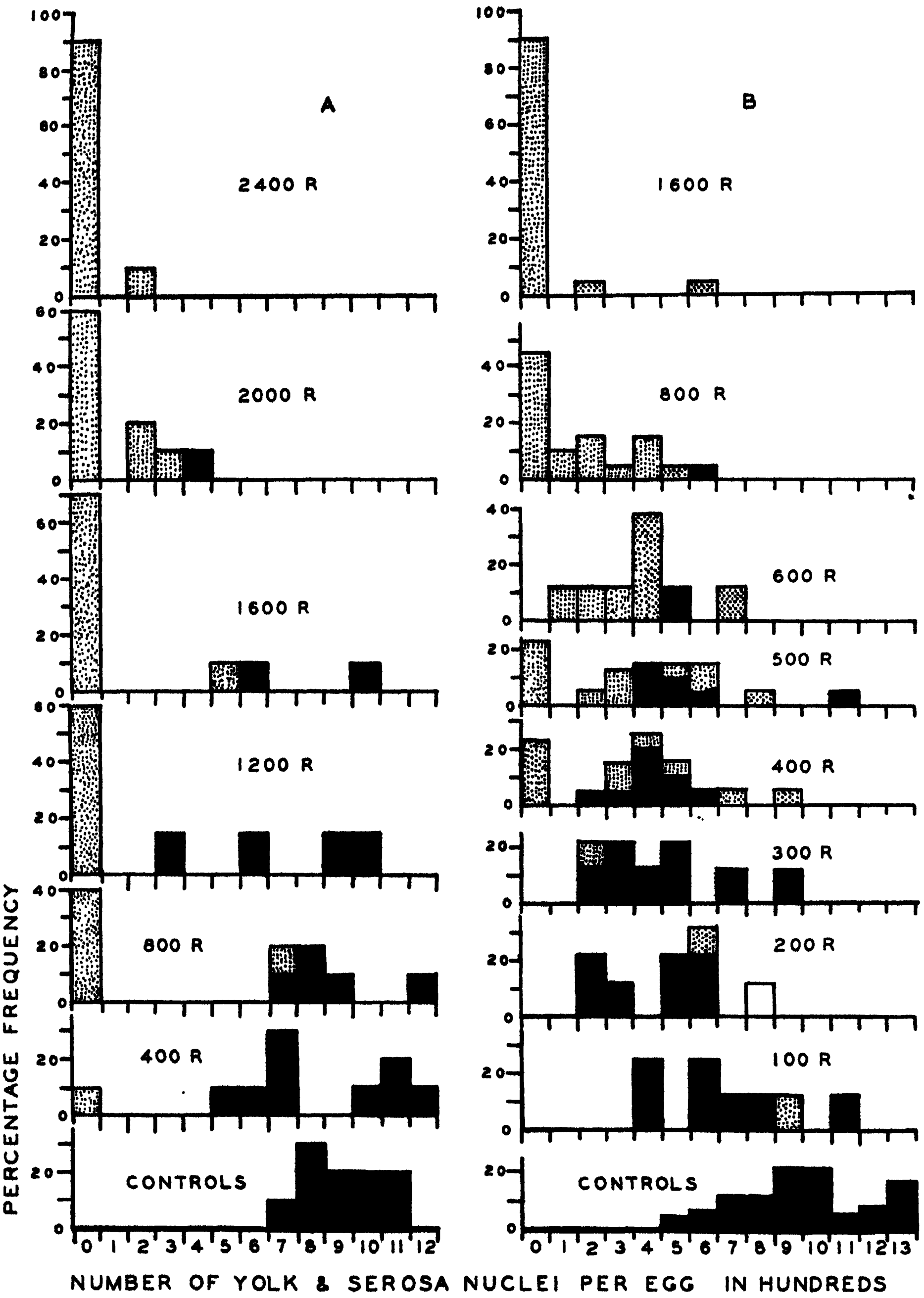

FIG. 6.—A frequency graph showing the effect of the radiation in decreasing the number of eggs containing the normal number of yolk and serosa nuclei. The solid columns indicate the percentage of the eggs containing embryos. The shaded areas represent the percentage number of eggs not containing an embryo. *A* is of eggs rayed when between 2 and 12 hours of age; *B* is of eggs between 12 and 24 hours of age when irradiated.

Typical serosa and yolk nuclei were formed between the fifth and seventh days in the control eggs as shown in Curve 1 of Figure 7. After a maximum of around a thousand nuclei was reached, the number remained about the same during the rest of the prediapause period.

One lot of eggs was irradiated with a dosage of 400 r, and the results are shown in Curve 2 of Figure 7. Individual variation was found, since some counts were as high as that of the controls whereas others were low. The general trend, as well as can be determined by inspection, seemed to indicate a slight delay in the attainment of the maximum number and for this peak to be a little lower than that of the controls. The effect of 500 r appeared to be more definite (Curve 3). In this case the maximum number was not reached until some 7 days after the controls, and the number of yolk and serosa nuclei finally formed was less than half of that of the controls. A dosage of 800 r (Curve 4) delayed the formation of the yolk and serosa nuclei still more and further reduced the number formed. Some eggs were irradiated with dosages of 1,600, 2,000, 2,500, and 3,200 r. With the exception of 1 egg (rayed 1,600 r), these failed to form any definite yolk and serosa nuclei. The eggs irradiated with dosages of 1,600 r and with 2,000 r were found at times to contain a few cells, but they were either undifferentiated or definitely injured.

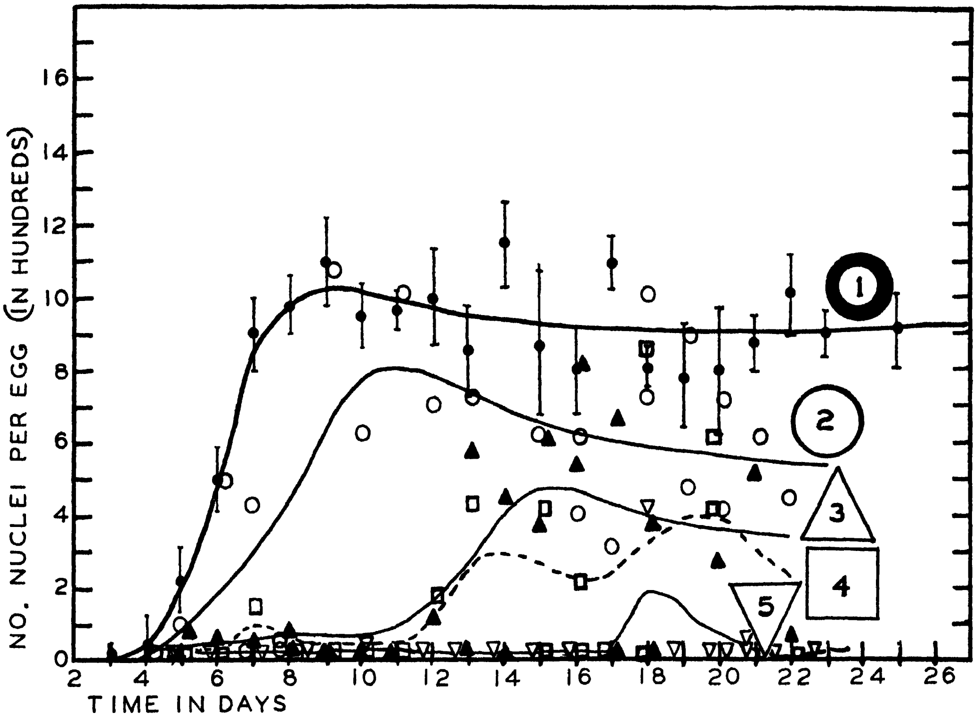

FIG. 7.—A graph showing changes in number of yolk and serosa nuclei of control and irradiated eggs. Curve 1 is through means and standard errors of 9 lots of control eggs. Curve 2 is of a lot of eggs irradiated with a dosage of 400 r. Curve 3 is of a lot of eggs rayed with a dosage of 500 r. Curve 4 is of eggs exposed to 800 r of radiation. A dosage of 1,600 r was given to the eggs of Curve 5.

DISCUSSION

The results indicate that irradiation on the first day may affect all the changes that normally take place during the prediapause development. These processes are dependent upon one another during the first day. This condition, if it occurs at all, must change very rapidly in *Drosophila*, since Henshaw (1935) states that mitosis goes forward apparently unmolested after irradiation, little or no differentiation takes place, and death is probably caused by unrestrained growth and lack of differentiation of the cells.

Ray (1938) has reported that irradiation on the first day prevents the subsequent formation of tyrosinase in the *M. differentialis* egg. He has found that dosages of 250–500 r lower the rate of tyrosinase production to such an extent that an additional 15 days is required for the concentration of the enzyme to reach the control level. Dosages of 1,000–2,000 r produce an inhibition of tyrosinase formation, from which there is little recovery. From these and other experiments he has concluded that the tyrosinase must be formed in the yolk or serosa cells rather than in the embryo proper. The results of the present investigation are in accord with his conclusion, as 1,000–2,000 r of radiation have been found to affect the development of the yolk and serosa cells with little or no recovery.

SUMMARY AND CONCLUSIONS

Eggs of *M. differentialis* have been irradiated on the first day of development and subsequent effects noted. The effects are failure to (1) form an embryo, (2) increase rate of oxygen consumption, (3) increase weight, and (4) form yolk and serosa cells. The developmental processes studied are closely related on the first day as a dosage of radiation heavy enough to affect one will also affect the others. The first effect to appear after irradiation is injury and destruction of undifferentiated cells. This effect appears within 24 hours after a heavy irradiation. The effects on the other processes appear on the fifth and sixth days.

LITERATURE CITED

BODINE, J. H. 1923. Hibernation in Orthoptera. I. Physiological changes during hibernation in certain Orthoptera. Jour. Exper. Zoöl., 37:457–76.

———. 1929. Factors influencing the rate of respiratory metabolism of a developing egg (Orthoptera). Physiol. Zoöl., 2:459–82.

———. 1932*a*. Hibernation and diapause in certain Orthoptera. II. Response to temperature during hibernation and diapause. *Ibid.*, 5:538–48.

———. 1932*b*. Hibernation and diapause in certain Orthoptera. III. Diapause—a theory of its mechanism. *Ibid.*, pp. 549–54.

EVANS, T. C. 1934. Respiration and visible development of individual grasshopper eggs following x-irradiation. Physiol. Zoöl., 7:556–71.

———. 1935. Variations in susceptibility to x-rays of *Melanoplus differentialis* eggs during development. *Ibid.*, 8:521–29.

———. 1936*a*. Qualitative and quantitative changes in radiosensitivity of grasshopper eggs during early development. *Ibid.*, 9:443–54.

———. 1936*b*. Use of roentgen radiation in analysis of metabolism in eggs of *Melanoplus differentialis*. Anat. Rec., Suppl. No. 1, 47:60.

———. 1937. Effects of roentgen radiation on embryonic organization and regulation in *Melanoplus differentialis* (Orthoptera). Physiol. Zoöl., 10:258–68.

———. 1938. Sequence of appearance of x-radiation effects. Anat. Rec., Suppl., 72:43.

HENSHAW, P. S. 1935. Changes in susceptibility of *Drosophila* eggs to x-rays. II. Correlation of biological activity and radiosensitivity. Radiology, **24**:438–43.

RAY, O. M. 1938. Effects of roentgen rays on the activation and production of the enzyme tyrosinase in the insect egg (Orthoptera). Radiology, **31**:428–37.

SLIFER, E. H. 1932. External morphology of grasshopper embryos of known age and with a known developmental history. Jour. Morph., **53**:1–21.

———. 1937. Origin and fate of the membranes surrounding the grasshopper egg. Quart. Jour. Micr. Sci., **79**:493–506.

1938. The formation and structure of a special water-absorbing area in the membranes covering the grasshopper egg. *Ibid.*, **80**:437–57.

SLIFER, E. H., and KING, R. L. 1934. Early stages in the development of grasshopper eggs of known age and with a known temperature history. Jour. Morph., **56**:593–601.

OXYGEN AS A FACTOR IN THE POLARITY OF CORYMORPHA PALMA

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BARTH (1938) has recently reported on the influence of oxygen in the reconstitution of *Tubularia*. The work of Child on the determination of polarity in cell aggregates (1928) and in stems of *Corymorpha* (1926, 1927) by the environmental differential between the upper free surface and the surface in contact strongly suggests that there also oxygen is a decisive factor. This presumption is supported by further work of Child and Hyman (1926), who found that respiration in *Corymorpha* is greatest in the hydranth and decreases basipetally over the naked stem region. Later Watanabe (1935) showed that the position of a future hydranth in a regenerating stem is marked out as a region of rapid methylene blue reduction several hours before the hydranth appears. In nature, too, where *Corymorpha* grows with its base imbedded in the mud and the hydranth extended freely into the water, it seems reasonable to assume the existence of a possible correlation between oxygen and polarized growth. In this paper I wish to present evidence that oxygen availability does actually affect hydranth formation and thereby influences the polarity of this organism. The work was suggested by Dr. Barth and was done during the summer of 1939 at the Scripps Institution of Oceanography. I am deeply indebted to the director, Dr. Harald U. Sverdrup, for the use of the laboratory; and to Dr. Marston C. Sargent and Mr. Percy S. Barnhart for many kind privileges.

METHOD

The specimens of *Corymorpha* used in these experiments were obtained during the "minus tides" of July at Mission Bay, where they may be found imbedded in the mud a few inches under water. They were about 3–4 cm. long. About 50 animals were obtained in all. The hydranths were cut off 1–2 mm. below the neck and discarded. Stem pieces averaging about 1.2 cm. in length were then snipped off 1–2 mm. above the perisarc-covered base. Basal as well as stem pieces were used. In the last experiment pieces were obtained from regenerated animals, since it was impossible to find fresh material. The temperature was approximately 21° C., except where otherwise stated below.

EXPERIMENTAL

Thirty-seven basal pieces were divided into two lots of 25 and 12, respectively. The first lot was allowed to regenerate in finger bowls of sea water. Within 24 hours they had healed at the cut surface and had secured themselves to the bottom of the dishes by means of holdfasts. Seventeen of the 25 had hydranths with rudimentary tentacles in 40 hours. Twenty-four had well-formed hydranths in 64 hours.

In the second lot the 12 bases were loosely capped over the cut surface with bits of glass tubing of $\frac{3}{32}$-inch bore, fused closed at one end. They were placed in finger bowls of sea water set on the sea-water table. These bases did not heal or regenerate at all during the course of the experiment, and later they disintegrated.

A procedure somewhat similar to the above was followed with 12 stem sections which were placed in pieces of submerged glass tubing of $\frac{3}{32}$-inch bore and $1\frac{1}{2}$ inches long, sealed at one end and loosely covered with cheesecloth at the other to prevent the stems from wandering out. These did not heal or regenerate. In comparison, 19 stems were divided into two lots of 10 and 9, respectively, and placed on cheesecloth stretchers in finger bowls of sea water. Flowing sea water, with oxygen content reduced to 3.71 cc. per liter, was siphoned into the dish containing 10 stems.[1] The water siphoned into the dish with 9 stems had a content of 5.07 cc. of oxygen per liter. In 21 hours at least two of the latter showed constriction of a hypostome. By 28 hours all of the stems at 5.07 cc. oxygen per liter and only half at 3.71 cc. had formed visible tentacle rudiments. In 34 hours 3 at 5.07 cc. and only one at 3.71 cc. had formed well-defined hydranths. At 44 hours fully developed hydranths with long tentacles and actively moving hypostomes were present in all at 5.07 cc. oxygen tension. This was true of only 5 out of 10 at an oxygen tension of 3.71. At 68 hours 3 out of the 10 in this lot still had hypostomes which were rather short and inactive.

In another experiment 12 stems were divided into two lots of 6 each and placed in glass-stoppered Erlenmeyer flasks of 125-cc. capacity. The flasks had been completely filled with aerated sea water, and nitrogen gas was bubbled through one for several minutes. Both were then sealed. By the end of 19 hours all the stems in the flask containing aerated sea water had healed and begun to form hypostomes. In 3 stems tentacle rudiments were present at 27 hours, and complete small hydranths were visible in 32 hours. One of these stems became bipolar during the next 10 hours. Compared with regeneration in normal sea water in the previous experiment, these show inhibition at this time, presumably due to consumption of oxygen by respiration. Between 42 and 52 hours, 2 additional stems had reconstituted, and, in all, 3 were bipolar and 2 unipolar. At 66 hours examination showed 5 out of 6 regenerated, but tentacles were still rudimentary in 2, indicating an insufficiency of oxygen for the completion of development. The flask was now opened, and the oxygen content was found to be 1.47 cc. per liter.

In the flask through which nitrogen had been bubbled the stems showed some healing at the ends but no further progress. When the experiment was terminated, chemical analysis showed that there was no free oxygen.

In a final experiment fragments were obtained from regenerated specimens. Four Erlenmeyer flasks were used. Nitrogen gas was bubbled through three of them for various lengths of time. Five basal and 4 stem pieces were placed in each flask. At 29 hours, owing to the clogging of the sea-water pipes, the temperature rose to, and remained at, about 26° C., and a few bubbles of gas appeared at the top of each flask. The results of this experiment must therefore be considered with caution. At 44 hours all the basal pieces had formed hydranths, whereas only 7 out of the total of 16 stems had begun to do so. All were retarded, in comparison with regeneration at the normal oxygen content of approximately 5 cc. per liter. At 52 hours all the basal pieces and 2 of the stems had fairly well-developed hydranths. The most significant difference was in the average number of tentacles developed by the basal pieces in the various flasks. These were 10.4, 8.4, 6.0, and 5.2, respectively, and were correlated with corresponding oxygen contents

[1] The oxygen content of the sea water was reduced by bubbling nitrogen through a reservoir of slowly flowing sea water. I am indebted to Professor F. B. Sumner for this suggestion.

of 1.69, 1.75, 0.96, and 0.54 cc. per liter, determined at 68 hours when the flasks were opened.

It may also be of some significance that the stem pieces were much more inhibited than the bases, indicating a possibly smaller oxygen requirement by the basal region.

DISCUSSION AND CONCLUSION

Although performed with a limited amount of material, the results of the above experiments all strongly suggest that oxygen is a determining factor in the formation of hydranths by regenerating portions of *Corymorpha* individuals.

It perhaps needs to be pointed out that bubbling nitrogen through sea water also probably reduces the CO_2 content. In connection with CO_2 variation, pH, too, may be affected. However, in the sealed flasks and in the glass tubes CO_2 is accumulated by metabolic activity. In this case pH (if affected beyond the buffering capacity of sea water at all) would also vary in a direction opposite to that in nitrogenated sea water. If metabolic products are presumed to play a part they, by the nature of the experimental procedures, would be accumulated in some cases and washed away in others. Therefore, while oxygen content was not the only variable in these experiments, it was the only factor with which hydranth formation can be consistently correlated.

As far as they go, these findings are in agreement with and support Barth's (1938) results with *Tubularia*. When considered together with evidence accumulating in other fields, such as the action of various growth-promoting substances upon root induction and the effects of CO_2, pH, etc., on polarity of *Fucus* (Whitaker, 1938), it seems reasonable to assume as a working hypothesis that, in organisms with labile polarity, pattern may be determined by factors which affect metabolic rate. Since Shapiro (1935) has demonstrated that *Arbacia* egg fragments containing the heavy granules have a higher respiratory rate than the lighter portions of the egg, it also becomes possible to interpret my results on the control of polarity in *Griffithsia* by centrifuging (Schechter, 1935) as a metabolic effect, i.e., as possibly due to higher respiratory rate at the centrifugal end. One is tempted to speculate further as to whether in organisms with fixed adult polarity the influences which emanate from organizing centers such as the lip of a blastopore during the labile period of embryonic development may not also be of a metabolic nature. Such an assumption would be supported by the multiplicity of agents which in experiments on induction have been shown to be able to act as organizers.

The evidence presented in this paper is in accord with Child's hypothesis of metabolic gradients. The experiments show that oxygen concentration influences the rate of hydranth formation. Since an existing hydranth exerts an inhibiting effect on hydranth formation by neighboring regions (Watanabe, 1935), the polarity of the animal is thereby determined.

SUMMARY

1. The regeneration of hydranths by sections of *Corymorpha* stem was noticeably inhibited at oxygen concentrations below 80 per cent of that of sea water which is in equilibrium with atmospheric air.
2. Retardation of oxygen diffusion by placing bases or stems in loosely fitting glass tubes submerged in sea water completely stopped reconstitution.
3. Sealing stems in comparatively large (125-cc.) volumes of sea water, from which most of the oxygen had been eliminated, completely stopped reconstitution.

LITERATURE CITED

BARTH, L. G. 1938. Oxygen as a controlling factor in the regeneration of *Tubularia*. Physiol. Zoöl., **11**: 179.

CHILD, C. M. 1926. Studies on the axial gradients in *Corymorpha palma*. III. Control and modification of polarity and symmetry in reconstitution by differential exposure. Biol. gen., **2**:771.

———. 1927. Modification of polarity and symmetry in *Corymorpha palma* by means of inhibiting conditions and differential exposure. Jour. Exper. Zoöl., **47**:343.

-. 1928. Axial development in aggregates of dissociated cells from *Corymorpha palma*. Physiol. Zoöl., **1**:419.

CHILD, C. M., and HYMAN, L. H. 1926. Studies on the axial gradients in *Corymorpha palma*. I. Respiratory, electric, and reconstitutional gradients. Biol. gen., **2**:355.

SCHECHTER, V. 1935. The effect of centrifuging on the polarity of an alga, *Griffithsia bornetiana*. Biol. Bull., **68**:172.

SHAPIRO, H. 1935. The respiration of fragments obtained by centrifuging the egg of the sea urchin, *Arbacia punctulata*. Jour. Cell. and Comp. Physiol., **6**:101.

WATANABE, Y. 1935. Physiological dominance in *Corymorpha palma* in relation to reconstitution and methylene blue reduction. Physiol. Zoöl., **8**:417.

WHITAKER, D. M. 1938. The effect of hydrogen ion concentration upon the induction of polarity in *Fucus* eggs. Jour. Gen. Physiol., **21**:833.

THE EFFECTS OF ACETYLCHOLINE, ATROPINE, AND NICOTINE ON THE ISOLATED HEART OF THE COMMERCIAL CRAB, CANCER MAGISTER DANA[1]

(Eight figures)

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THE effects of acetylcholine and of atropine on the arthropod heart have been studied by Welsh (1939*a*, 1939*b*) working with the hearts of *Carcinus* and *Panulirus*, by Hamilton (1939) working with the heart of the insect *Melanoplus*, and briefly by the writer and students (1940) working with the heart of the crayfish *Astacus*. Carlson (1906) nicotinized the heart of *Limulus*, in which ganglia and muscle can be separately treated. Yeager and Gahan (1937) and Yeager (1938) studied the effects of nicotine on the isolated hearts of the insects *Periplaneta* and *Prodenia*, Hamilton studied its effects on the grasshopper heart, and the writer made preliminary tests on the heart of the decapod *Astacus*.

There has been found to be considerable variation in the response of various arthropod hearts to acetylcholine; further information on its effect on the hearts of other forms as well as on its mode of action is needed. To the writer's knowledge no study has been made of the effects of nicotine on the decapod heart other than his brief tests mentioned above, which seemed to present evidence that the action of acetylcholine on crustacean hearts is "muscarine-like" rather than "nicotine-like." It seemed worth while, therefore, to carry on more extensive tests with these drugs. For this purpose the heart of the large commercial crab of the West coast, *Cancer magister*, was used.

The large size of this heart was found to be of advantage in making the perfusion studies. It was easily prepared for isolation and perfusion by simply cutting into the animal from the ventral surface after it had been allowed to bleed and removing musculature, digestive organs, and gonad. The heart could then be seen within the pericardium attached to the carapace by its ligaments. The carapace was then trimmed away leaving a small, easily manipulated section to which the heart was attached. Lastly, the pericardium was removed from the surface of the heart, and the heart flushed out with perfusion fluid via the aperture left by the removal of the descending vessel. If the heart had stopped during manipulation, flushing by this method and the consequent return of internal pressure quickly restored the normal beat. A small incision was made just proximal to one or the other of the ventral anterolateral alae cordis, the cannula inserted and tied in. A thread was tied around the posterior tip of the heart just anterior to the median posterior ala and including the posterior aorta. The heart was then suspended by a thread between cannula and writing-arm after the remaining ligaments and vessels had been cut.

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[1] The writer wishes to express his thanks to Dr. Frank Weymouth of Stanford University and Dr. John Welsh of Harvard University for aid and advice during this work.

For perfusion a simple constant-level chamber was designed, based, with modifications, on that used by Welsh (1939*a*) for the heart of *Carcinus*.

After some experimentation a perfusion fluid was arrived at which closely resembled that used by Pantin (1934) and by Welsh:

NaCl	0 53 M	1,000 cc.
KCl	0 53	25 cc.
$CaCl_2$	0.53	30 cc.
$MgCl_2$	0 53	25 cc.
$NaHCO_3$	1 00	2 cc.
Dextrose		1 gm.

Experiments were carried out in a controlled-temperature room with the temperature at 15° C.

ACTION OF ACETYLCHOLINE

It was determined, just as had been in the heart of *Astacus in situ*, that different hearts responded quite differently to the mediator. The threshold of sensitivity varied between 1:10,000,000 and 1:1,000,000 in most uneserinized hearts. Some hearts, however, showed no acceleration or increase in amplitude until concentrations as high as 1:100,000 were administered. Generally, acetylcholine in above-threshold concentrations produced a more or less marked increase in amplitude and frequency. If a heart had been beating irregularly, threshold concentrations regularized and slightly accelerated the rhythm. In certain hearts, however, acetylcholine produced no marked increase in frequency or amplitude but produced an increase in tonus which was reflected by a rise in level of the recording (Fig. 1). High concentrations had been shown by Welsh to produce in *Carcinus* such a rapid firing of the pacemaker cells that the heart was unable

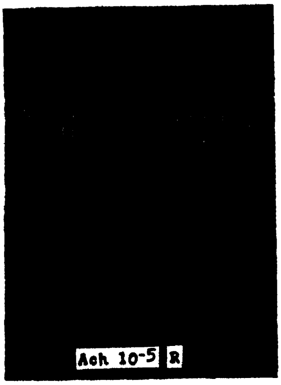

FIG. 1.—Effect of acetylcholine 1:100,000

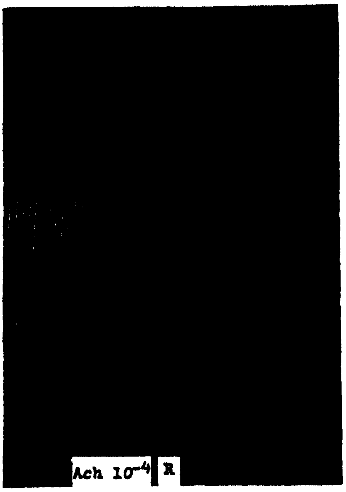

FIG. 2.—Effect of acetylcholine 1:10,000

to recover after each contraction, and a condition of systolic tetany followed. Such high concentrations as this were not tested on hearts which responded to low concentrations with the usual increase in frequency and amplitude, but in a heart which had responded by a tonus increase alone a solution of 1:10,000 produced such a marked increase in tone that the heart went into systolic stoppage. That this was not due to rapidly succeeding discharges from the pacemaker and was the result of a marked tonus increase can be seen in Figure 2.

As has been demonstrated in the hearts of other Crustacea, eserine increases the sensitivity of the heart of *Cancer* to acetylcholine; treatment with eserine 1:100,000 for 20 minutes would increase the sensitivity to the mediator from ten to one hundred times.

ACTION OF ATROPINE

This drug in concentrations as low as 1:20,000 abolished completely the effects of subsequently administered above-threshold concentrations of acetylcholine.

ACTION OF NICOTINE

Nicotine at a concentration of 1:20,000 produced in the isolated *Cancer* heart an increase in frequency and amplitude. This effect was not immediately reversible, for the heart would return approximately to its original beat only after some 15–20 minutes of washing with perfusion fluid.

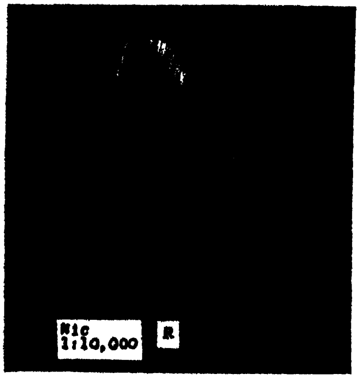

FIG. 3.—Effect of nicotine 1:10,000

FIG. 4.—Effect of nicotine 1:4,000

The effect of nicotine 1:10,000 was interesting. In certain tests this concentration produced merely an increase in amplitude and frequency much as did nicotine 1:20,000. In other tests, however, it brought about systolic tetany after a brief and very marked increase in frequency accompanied by a rise in tone (Fig. 3). There was evidence in one such test that a certain "conditioning" to the drug existed, for a rapid and deep beat started again before the reintroduction of perfusion fluid into the heart.

Nicotine in higher concentrations (1:4,000, 1:3,000) had varying effects. In certain tests (Fig. 4) a rise in tone or an increase in amplitude and frequency was followed by a decrease in frequency or irregularity of beat. In other tests the depressant effects of such concentrations following an increase in frequency did not become evident, but in spite of 20 minutes of washing with perfusion fluid the amplitude steadily decreased.

In most such tests there appeared a transitory rise in tone; however, the beat would return after a time to the same level with a slightly decreased amplitude.

In all tests with nicotine the effects of the drug were found to be somewhat permanent; tests with higher concentrations on a heart which had previously been treated with lower concentrations or vice versa did not bring about the same effect as treatment of a fresh heart, since the effects were never completely reversible.

ACTION OF NICOTINE AND ACETYLCHOLINE

A heart was brought to systolic stoppage with nicotine 1:10,000; and the beat shortly thereafter, in spite of washing with perfusion fluid, steadily became weaker and slower (apparently because of the depressing effect of the nicotine). Treatment with acetylcholine 1:100,000 brought about a transitory increase in frequency and amplitude (Fig. 5). A number of hearts which had previously been treated with nicotine 1:4,000 responded to acetylcholine 1:100,000 with the usual increase in frequency and amplitude. In a test in which continuous treatment with nicotine 1:4,000 had produced a very irregular beat, acetylcholine 1:10,000 had a marked regularizing effect which was followed by stoppage (Fig. 6). The irregular beat returned after brief washing with perfusion fluid. A heart was treated with acetylcholine 1:100,000, which was found to bring about a rise in tone without an accompanying increase in frequency. Later treatment with nicotine 1:3,000, which brought about an increase in amplitude, did not prevent the appearance of the same rise in tone when the acetylcholine was reintroduced (Fig. 7).

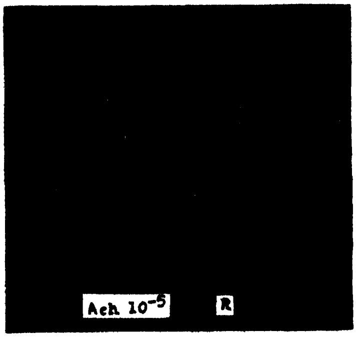

FIG. 5.—Effect of acetylcholine 1:100,000 after treatment with nicotine 1:10,000.

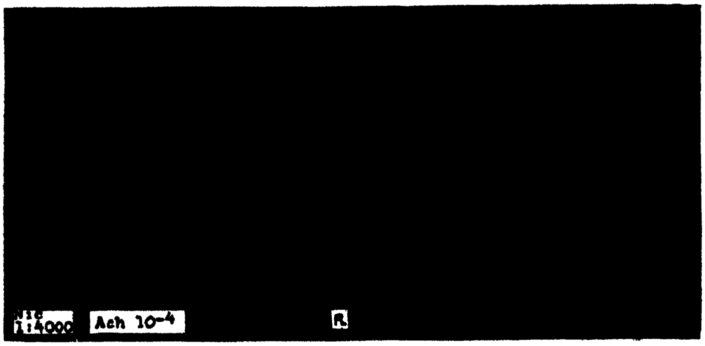

FIG. 6.—Effect of acetylcholine 1:10,000 after treatment with nicotine 1:4,000

DISCUSSION

The tests with acetylcholine which brought about a tonus change in the heart with no frequency increase would seem to indicate that the mediator works on the contractile mechanism as well as on the pacemaker and may work quite independently upon each.

But the effects on the contractile mechanism seem to become apparent only when high concentrations are used.

For some time there has been a question in the minds of workers as to whether the effect of acetylcholine on the arthropod heart is nicotine-like or muscarine-like. This writer has stated (1940) that the effects on the crayfish heart seemed to be muscarine-like since they were abolished by atropine; furthermore, there seemed to be no resemblance between the effects of nicotine and acetylcholine. But the nicotine tests on which these tentative opinions were based were brief and inconclusive. Not a wide enough variety of concentrations were used; and it is also questionable whether the drugs reached the intrinsic ganglion without dilution, since the heart was autoperfused. Nicotine 1:10,000 was found to bring about an increase in amplitude but a decrease in frequency. It is difficult to interpret these results in the light of those obtained on the isolated *Cancer* heart. It is possible that for the *Astacus* heart a concentration as high as 1:10,000 had a depressing effect on the pacemaker, but one would have expected an initial stimulation.

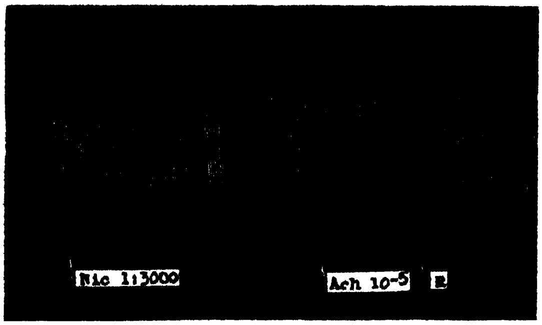

FIG. 7.—Effect of acetylcholine 1:100,000 after treatment with nicotine 1:3,000.

In the heart of *Cancer* it was found that the effect of acetylcholine is abolished by atropine. However, it was also found that nicotine 1:10,000 brings about systolic stoppage in the *Cancer* heart, at least for a brief period, by stimulating the pacemaker to discharge so fast that the muscle is unable to recover and systolic tetany results. This is strikingly similar to the response of the heart of *Panulirus* to acetylcholine 1:10,000 (Welsh, 1939*b*). Furthermore, in *Cancer* nicotine in lower concentrations causes a marked increase in frequency and amplitude just as does acetylcholine, though the effects of the former are somewhat cumulative and irreversible. There is evidence, therefore, that the two substances work in much the same manner on the isolated and perfused heart. Carlson (1906) in his classic experiments on the heart of *Limulus*, in which ganglion and muscle can be separately treated, was the first worker to demonstrate that nicotine in dilute solutions has a primary stimulating effect on the ganglion of an arthropod heart and that at higher concentrations the drug produces irregularity in rhythm, depression, and paralysis. He showed that in lower concentrations nicotine has little effect on the muscle itself. It seems probable, therefore, that in the crustacean heart perfused with nicotine the intrinsic ganglionic cells are stimulated.

The innervation of the hearts of various Crustacea has been extensively discussed by Alexandrowicz (1932). Welsh (1939*b*) says:

> Alexandrowicz has shown that, in spite of the small number of nervous elements, the morphological situation is likewise complex. Transection of the nerve trunk in the mid-region stops the regular beat of the heart. The ganglion may then be considered the pacemaker of the heart.... and .... any substance which (1) affected the pacemaker activity of the ganglion cells, (2) facilitated transmission at myoneural junctions, or (3) acted on muscle cells directly might modify the frequency and amplitude of beat.

TABLE 1

EFFECTS OF DRUGS ON ARTHROPOD HEARTS

Animal	Acetylcholine Threshold	Eserine	Acetylcholine after Atropine	Nicotine	Acetylcholine after Nicotine
Carcinus sp. (Welsh, 1939*a*)	10^{-8}	Not tested	Abolished		
Panulirus argus (Welsh, 1939*b*)	10^{-10} 10^{-9}	10^{-5} regularized beat or increased frequency; subsequent acetylcholine not tested	Abolished		
Limulus polyphemus (Carlson, 1906)				*Ganglia:* Low concentrations gave stimulation; high gave stimulation followed by depression and paralysis *Muscle:* Low concentrations had little effect	
Astacus trowbridgei (Davenport, Loomis, and Opler, 1940)	10^{-10}	10^{-5} regularized or gave initial increase in amplitude followed by decrease in amplitude and increase in frequency; subsequent acetylcholine not tested	Abolished	1 : 10,000 gave increase in amplitude and decrease in frequency; 1 · 5,000 decrease in amplitude and irregularity of beat	
Cancer magister (present work)	10^{-7}	Some hearts showed increase in frequency, some no effect; 10^{-5} for 20 minutes lowered threshold to acetylcholine approximately ten times	Abolished	1 : 20,000 gave increase in amplitude and frequency; 1 : 10,000 increase in amplitude and frequency or increase in frequency to systolic stoppage; 1 : 4,000, 3,000 initial stimulation of frequency and amplitude followed by depression	High concentrations do not abolish effect of subsequently administered acetylcholine
Melanoplus differentialis (Hamilton 1939)	1×10^{-14} M (2.26×10^{-15})	Not tested	Abolished	1 : 100 to 1 : 1,000,000 gave increase in amplitude and slight decrease in frequency; low concentrations in some hearts gave initial increase in frequency	

TABLE 1—*Continued*

Animal	Acetyl-choline Threshold	Eserine	Acetyl-choline after Atropine	Nicotine	Acetylcholine after Nicotine
Periplaneta americana and *Prodenia eridania* (Yaeger and Gahan, 1937)				Low concentrations gave initial stimulation in frequency without depression; intermediate concentrations, stimulation followed by partial depression; and high concentrations, stimulation followed by depression and paralysis; concentrations giving initial stimulation increased tone of heart	

A characteristic nicotine-like effect of acetylcholine in vertebrates is stimulation of the cells of sympathetic ganglia. There is evidence that acetylcholine has this stimulatory effect on the ganglion of the crustacean heart, because of the similarity of its effect to that of nicotine, which we know stimulates arthropod ganglionic cells. But in vertebrates muscarine-like effects are abolished by atropine, just as is the effect of acetylcholine on the *Cancer* heart.

It is possible to formulate a hypothesis to explain this anomolous situation in the *Cancer* heart. Acetylcholine may have a nicotine-like effect on the ganglion cells and may have a muscarine-like effect at the myoneural junction (Fig. 8). If, as in the vertebrates, nicotine-like effects are abolished by high concentrations of nicotine, then in the light of this "two-effect" hypothesis the stimulatory effects seen in Figure 5, in which the ganglionic cells have been depressed by high concentrations of nicotine, cannot be a result of stimulation of these cells but must be the effect of the introduced acetylcholine on the myoneural junction or the muscle directly.

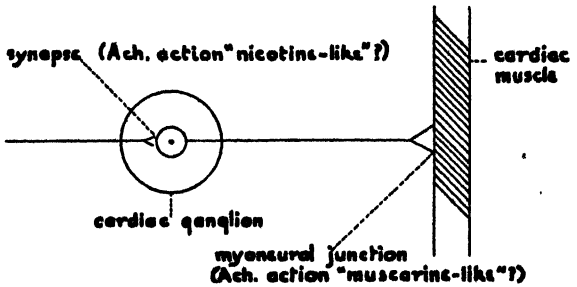
synapse (Ach. action "nicotine-like"?)
cardiac muscle
cardiac ganglion
myoneural junction (Ach. action "muscarine-like"?)

FIG. 8.—Hypothetical mediation mechanism in the ganglion and muscle of the heart of *Cancer*.

It is apparent that further experiments must be carried out to test this hypothesis.

Table 1 presents a brief summary of the effects of the drugs discussed on arthropod hearts. It can be seen that the effect of nicotine on the hearts of *Periplaneta* and *Prodenia* (Yeager and Gahan, 1937) is somewhat similar to its effect on the heart of *Cancer*. However, in the roach heart systolic stoppage is brought about by nicotine by a steady increase in tone without a marked increase in frequency (Yeager, 1938, Fig. 7). It can be seen that in the heart of *Melanoplus* Hamilton (1939) noted little increase in frequency following treatment with nicotine, and that the heart of this insect is remarkably sensitive to acetylcholine.

LITERATURE CITED

ALEXANDROWICZ, J. S. 1932. The innervation of the heart of the Crustacea. I. Decapoda. Quart. Jour. Micro. Sci., **75**:181.

CARLSON, A. J. 1906. On the point of action of drugs on the heart with special reference to the heart of *Limulus*. Amer. Jour. Physiol., **17**:177.

DAVENPORT, D.; LOOMIS, J. W.; and OPLER, C. F. 1940. Notes on the pharmacology of the hearts of *Ariolimax columbianus* and *Astacus trowbridgei*. Biol. Bull., **79**:498.

HAMILTON, H. L. 1939. The action of acetylcholine, atropine, and nicotine on the heart of the grasshopper (*Melanoplus differentialis*). Jour. Cell. Comp. Physiol., **13**:91.

PANTIN, C. F. A. 1934. On the excitation of crustacean muscle. I. Jour. Exper. Biol., **11**:11.

WELSH, J. H. 1939*a*. Chemical mediation in Crustaceans. II. The action of acetylcholine in nervous tissues and its action on the decapod heart. Jour. Exper. Biol., **16**:198.

———. 1939*b*. Chemical mediation in Crustaceans. II. The action of acetylcholine and adrenalin on the isolated heart of *Panulirus argus*. Physiol. Zoöl., **12**:231.

YEAGER, J. F. 1938. Mechanographic method of recording insect cardiac activity, with reference to effect of nicotine on isolated heart preparations of *Periplaneta americana*. Jour. Agric. Res., **56**:267.

YEAGER, J. F., and GAHAN, J. B. 1937. Effects of the alkaloid nicotine on the rhythmicity of isolated heart preparations from *Periplaneta americana* and *Prodenia eridania*. Jour. Agric. Res., **55**:1.

OBSERVATIONS ON RODENTS IN HERTER'S TEMPERATURE GRADIENT

(One figure)

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HERTER described in 1924 a new temperature-gradient apparatus (*Temperaturorgel*) which was intended particularly for observations on the modal or so-called preference temperature of rodents. This new gradient differs from the older type used by the same author by having angled thermometers inserted within a bottom layer of aluminum. The readings thus obtained are obviously those of the bottom temperature. To correlate those results with the modal temperature of the rodents seemed justified, as these animals cling to the bottom with a very broad ventral surface.

Herter was not unaware of the fact that there exists a rather considerable difference between the temperature of the aluminum layer and the temperature of the air above it. But in his experiments the modal temperature of the rodents remained unchanged when the horizontal differences of air temperature were eliminated and equalized by ventilation of the interior of the gradient for short periods. In addition, mice with blackened belly (+) and those with depilated belly (−) showed an increase or decrease of about 1° C. in their modal temperature as compared with normal individuals. Herter, therefore, concluded that the mice are directed in their thermo-orientation only by the soil temperature.

According to our experiments on the scale of activity, the instantaneous death temperature of the recently born (up to a fortnight old) *Microtus guentheri* D. and A. fluctuates between 38.5° and 39.5° C. Whereas the modal temperature of the same ages of this vole, as it was measured by Herter's procedure, ranges between 38.5° and 39.0° C. The contradiction of these results is obvious and definitely excludes the assumptive identity of soil temperature and modal temperature.

A simple consideration may help to understand this contradiction. People in a room of 16°–20° C. like to press their backs to a stove of Dutch tiles with a surface temperature of 37°–41° C. This common experience would not make anyone assume that 37°–41° C. is the modal or preference temperature of *Homo sapiens* L. Despite the partially justified criticisms of Gunn, the readings of the air temperature at a distance from the soil which is in relation to the size and the height of the species studied are the relatively safest indicators for studies of modal temperature. This method was, therefore, tried in comparison with Herter's method.

The presence of air thermometers within the gradient interferes and disturbs the observations on the modality of the rodents. Air temperatures were therefore measured at different heights in the empty apparatus, whereby the agreement of the general course of temperature during the observation was ascertained by comparing the readings of the soil thermometers. The conversion table, calculated from these observations for an air temperature at 2.5 cm. above the soil, is shown graphically as Figure 1.

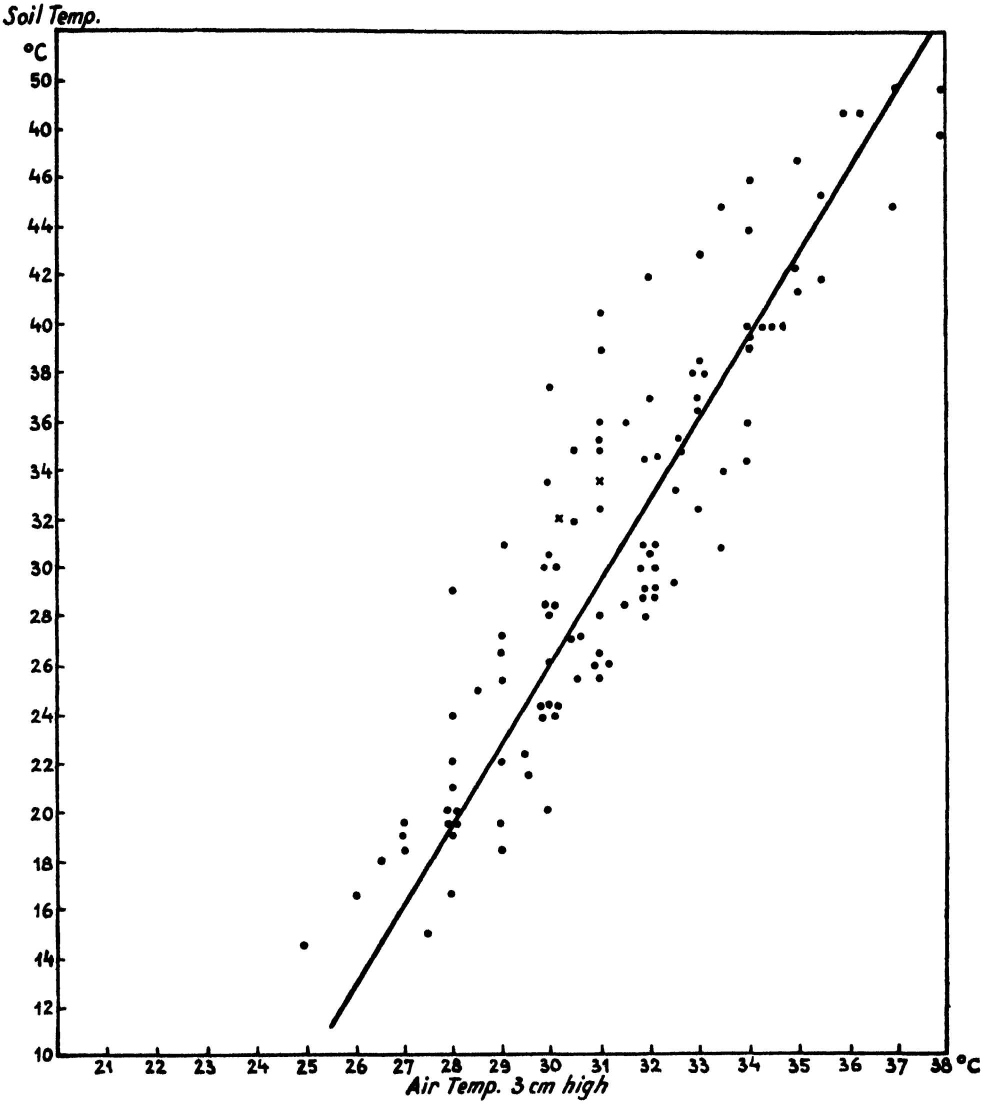

Fig. 1.—Conversion table of soil temperatures into air temperatures

EXPERIMENTS

All readings on the soil thermometers were converted by means of these tables. The results concerning the adults of 11 different species of rodents are compiled in Table 1 (M = statistical average; m = mean error).

The averages of the modal temperature of all these rodent species seem to be rather close, much closer than in Herter's publications. The analysis as to whether the differences are significant or not is made by the formula

$$x = \frac{M_1 - M_2}{\sqrt{(m_1)^2 + (m_2)^2}} .$$

TABLE 1

MODAL TEMPERATURE OF SOME RODENTS OF PALESTINE

No.	SPECIES	WEIGHT IN GRAMS	*n*	*M* (HERTER'S METHOD)	*M*±3*m* (OUR METHOD)	TEMPERATURE (° C.)												
						25	26	27	28	29	30	31	32	33	34	35	36	37
1 .	*Cricetulus migratorius cinerascens* Wag.*	28–36	181	31 07	31 05±0 252		. .	.	3	14	*36*	*61*	*53*	13	1	. . .		. .
2 .	*Cricetus auratus* Wtrh.†	66–85	253	32 73	31 51±0.243	. .		. . .	3	8	*52*	*49*	*90*	*39*	9	3	. .	.
3 .	*Rattus norvegicus* Erxl. (white)†	117–183	252	33 20	31 70±0 303	. .	. .	3	5	13	*33*	*50*	*73*	*47*	17	10	1	. .
4	*Mus musculus musculus* L (white)†	12–17	249	32 48	31 44±0 246		.	4	4	14	24	*56*	*112*	29	5	1	. . .	. .
5. .	*M. musculus gentilis* Brants	10–15	262	34 03	31 84±0 096	.	. .	. .	.	.	7	*56*	*172*	27	. .	. . .	. . .	
6.	*Acomys cahirinus dimidiatus* Cretzsch.	20–60	205	32 50	31 43±0 222		. .		.	14	18	*56*	*96*	16	5		.	.
7	*Dipodillus dasyurus* Wagn	22	62	33 35	31 60±0 378		.				10	*17*	*24*	10	1	.	.	
8. .	*Meriones tamaricinus tristrami* Thos.	20–120	260	30 85	30 94±0 375	. .	5	5	30	28	33	*36*	*61*	*43*	15	4	.	. . .
9 .	*Microtus guentheri* D. and A	15–80	398	31 66	31 23±0 267		.	11	23	25	*77*	*66*	*104*	*63*	27	4	2	.
10 .	*Cavia cobaya* L †	221–406	252	35 82	32 20±0 915	. .	. . .	.	2	7	16	28	*77*	*84*	34	4	.	. .
11	*Spalax ehrenbergi* Nhrg	. .	134	32.04	31 28±0 294	. .		.	.	5	*35*	*31*	*45*	16	2	.	. .	. .

* Experiments performed by Mr. Bindeman.

† Experiments performed by Mr. Wigodski.

TABLE 2

STATISTICAL ANALYSIS OF MODAL TEMPERATURES AS SHOWN IN TABLE 1*

Species Related	*x*	Species Related	*x*
5:1	9.3	10:1	8.3
5:2	3 7	10:2	5.0
5:3	1 2	10:3	3.3
5:4	4.5	10:4	5.5
5:6	5 1	10:5	3.1
5:7	1 9	10:6	5 8
5:8	6 9	10:7	3.6
5:9	6.6	10:8	7.6
5:10	3.1	10:9	6.8
5:11	5.4	10:11	2.8
Average	4.8	Average	5.2

* The value of *x* for the white rat and *Meriones*, e.g., is 4.7 (results are regarded as significantly different if *x* is larger than 3.0).

If *x* is larger than 3, the differences are regarded as statistically significant. The calculations given in Table 2 show that there is no doubt that most of the species have a significantly different specific modality. The recorded numbers for the different species are taken from the preceding table.

Three of the species used by us have also been studied by Herter. A comparison of the results obtained by both authors with Herter's method of calculation indicates a rather surprising degree of agreement for two species and a significant difference in the third one, as shown in Table 3.

These results show that Herter's new apparatus is quite reliable for obtaining comparable results in rodents. With regard to white mice the difference is possibly a constitutional one.

TABLE 3

COMPARISON OF TEMPERATURE MODALITIES WITH THOSE OBTAINED BY HERTER (1924)

Species	Herter's Results (° C)	Our Results (° C.)
White rat	33·23	33 20
White mouse	34 63	32 48
Cavia cobaya	36 22	35 82

TABLE 4

MODAL TEMPERATURE OF *M guentheri* AT DIFFERENT AGES

AGE IN DAYS	*n*	*M* (HERTER'S METHOD)	*M*±3*m* (OUR METHOD)	MODALITY AIR TEMPERATURE 2 5 CM ABOVE SOIL (° C)												
				27	28	29	30	31	32	33	34	35	36	37	38	39
2	107	38 27	33 32±0 303					4	19	*37*	*34*	12	1			
4	113	38 52	33 21±0 243					3	15	*58*	*29*	8				
6	118	38 69	32 94±0 429				2	4	*25*	*47*	*27*	10	4	1		
10	164	37 81	35 00±0 336			1	4	4	12	5	9	*49*	*64*	15	1	
15	135	36 54	34 61±0 339				1		2	8	*57*	*61*	14	3		
21	128	33 28	33 67±0 342					8	21	14	*56*	21	7	1		
40	157	33·23	33 71±0 342					5	*33*	*29*	*47*	*30*	5	7	1	
73	142	32 12	33·31±0 426			1	5	18	25	19	*40*	23	8	1	2	
81	186	35 17	34 24±0 315					5	17	*31*	*55*	*46*	18	13	1	
105	118	30·72	30 93±0 477	1	10	10	24	21	*37*	10	3	2				
209	136	32 79	31 63±0 471	2	8	8	10	19	*31*	*32*	13	2	2			
300	144	31 48	31 03±0 426	8	5	7	*34*	*26*	*36*	21	7					

The difference between the modality of young and old rodents is greatly reduced by the conversion of the soil temperatures into the air temperatures. Extensive experiments on *M. guentheri* yielded the results listed in Table 4.

Herter's statement that the modality decreases from birth to old age is confirmed in principle. The difference is, however, reduced from 8.15° C. (38.37°–30.72° C.) to 2.35° C. only (33.82°–31.47° C.). The higher modality between 10 and 15 days of age, i.e., shortly before and after weaning, may be caused either by the increased locomotor ability or by a lower degree of thermo-orientation in the neonatal rodents.

The difference between young and recently matured adults is small and insignificant, whereas the difference between either or both groups and old adults is highly significant, as shown in Table 5.

DISCUSSION

There is no doubt, as has been pointed out, that the results obtained with mammals in Herter's modified apparatus have no direct relation to the specific "comfortable" temperature. But, on the other hand, correlation between air temperature and modality yields principally identical results. The results obtained for 11 species of rodents are, practically, all significantly different with regard to their temperature modality. And this in spite of the fact that the range is much narrower than in Herter's method, where the readings are correlated to soil temperature.

The theoretical background is as follows: When poikilothermic animals are exposed to a temperature gradient, the body temperature and the intensity of all physiological processes are changed. No thermic sense organs are known, but it is reasonable to assume that the modal temperature is the so-called comfortable temperature. We are, unfortunately, unable, so far, to understand the mechanism of thermo-orientation in poikilothermic animals. There seems a possibility of connecting it, partially at least, with the water metabolism of the body tissues (Bodenheimer, 1931).

TABLE 5

STATISTICAL ANALYSIS OF THE EFFECT OF AGE ON MODAL TEMPERATURE OF *M. guentheri*

Age in Days	$M \pm 3m$ (° C.)	$\frac{M_1-M_2}{m \text{ Diff.}}$
1–10	33 82±0 201	1:2= 1.23
15–81.. . . .	33 92±0.132	1:3=23 3
105–300....	31 22±0 264	2:3=27.3

The thermo-orientation of mammals seems to be of an entirely different character. The more or less constant body temperature of homiothermic animals is a very unreliable guide, as we know from daily experience. Our thermo-orientation is highly influenced by contrast effect. Mammals possess poor powers of estimating surrounding temperatures. They can distinguish and avoid extreme temperatures and come to rest in the least stimulating of available temperatures. With man this zone of comfort fluctuates between 16° and 20° C. But this "comfortable" temperature is not the temperature obtained in Herter's apparatus. The rodents dilate the belly as far as possible and press it flat on the surface of the soil, just as men press the back against a stove with a surface temperature twice as high as the comfortable temperature, one entirely unfit for permanent sojourn. This is confirmed by our observation that the modality obtained in the temperature gradient for young *Microtus* is identical with their instantaneous death temperature.

What the experiment really proves is (1) the presence of thermo-orientation in rodents and (2) significant specific differences in the rodents studied. Herter's method of using the readings of the soil temperature is preferable to the conversion of these readings into air temperatures because (1) the directive temperature stimulus comes from the soil surface and not from the air temperature and (2) the range of fluctuation is larger for soil temperatures than for air temperatures and the readings therefore more convenient. Since this paper was written, a study by N. Kalabuchov (1939) has appeared, from which further details can be learned.

Our experiments were performed in an apparatus which was built exactly after Herter's indications. But Kalabuchov changed its proportions (length, 120 cm. versus 60 cm.; breadth, 15 cm. versus 10 cm.). This apparatus seems better suited to observations of rodents than does the rather short apparatus used by Herter and by myself. But, un-

TABLE 6

EFFECT OF LATITUDE AS MODAL REACTION IN A TEMPERATURE GRADIENT

Localities	No of Specimens	No of Readings	Modal Temperature (° C)
Charkov	18	900	23.24
Moscow	17	850	26.44
Rostov	11	550	27 82
Pre-Caucasus	10	500	29 17

TABLE 7

MODAL TEMPERATURES FOR RODENTS AS DETERMINED TO DATE

SPECIES OF RODENTS	MODAL TEMPERATURES AS DETERMINED BY—		
	Herter	Bodenheimer	Kalabuchov
Eliomys quercinus	39 02±0 67		
Muscardinus pulcher	38 40±0 41		
M. avellanarius	38 45±0 29		
Glis glis	36 35±0 29		
Citellus pygmaeus			30 85
C. suslica			31 41
Cricetus cricetus	29 41±0 31		
C. auratus		32 73	
Cricetulus migratorius		31 07	
Mus musculus musculus	37 36±0 12		
M. m. musculus (white)	34 63±0 12	32 48	
M. m. gentilis		34 03	
M. m. azoricus	37 43±0 22		
M. wagneri rotans	33.85±0 13		
M. spicilegus	35 54±0 16		
Rattus norvegicus	35 06±0 18		
R. norvegicus (white)	37 23±0 27	33 20	
Micromys minutus	37 02±0 24		
Apodemus agrarius	36 14±0 22		
A. flavicollis	33 76±0 20		20 54
A. sylvestris	34 64±0 19		26 07
Evotomys glareolus	32 32±0 20		
Acomys dimidiatus		32 50	
Diplodillus dasyurus		33 25	
Meriones tristrami		30 85	
Microtus arvalis	35 05±0 30		
M. guentheri		31 66	
Arvicola terrestris	28 15±0 45		
Pitymys savii	29 39±0 43		
Cavia cobaya	36.22±0 19	35 82	
Spalax ehrenbergi	...	32 04	

fortunately, this modified apparatus gives entirely different results. This may be learned from the values obtained on two species of *Apodemus*, studied by both Herter and Kalabuchov. It seems, therefore, highly desirable, in order to obtain comparable results, that all students agree on one type of temperature gradient.

Kalabuchov makes the very interesting statement that the modal temperature increased in specimens of *A. silvaticus* L. from different latitudes, increasing from north to south, as shown in Table 6. The differences for another species (*A. flavicollis* Melch.) from Leningrad and Charkov were slight (only 20.34° as compared with 20.73° C.). Similar observations on poikilothermic animals have been made by Krumbiegel (1932, *Carabus*) and Lütfi (1936, *Lacertilia*). On the other hand, the three species of rodents (all domesticated species) studied both by Herter and by myself show a close agreement. This agreement is more remarkable as the climates of Berlin and Jerusalem differ more widely than those of the Russian localities among themselves. It is very desirable that such experiments should be continued with identical apparatus, in order to make certain that the results obtained are strictly comparable. Table 7 compiles the experimental results, so far obtained, on the modal temperature of rodents.

CONCLUSIONS

1. Observations of rodents in a temperature gradient show a distinct and specific thermo-orientation.
2. This thermo-orientation is directed by the soil temperature.
3. The modal temperature of rodents, as obtained in the temperature gradient, is not comparable with results obtained by the same method for poikilothermic animals. It is about twice as high as the "comfortable" temperature of each species.
4. For orientation on the specific differences of the thermo-orientation in different rodents the observation in the temperature gradient gives satisfactory results. No further conclusion should be drawn, and no value ascribed to the absolute temperatures of the readings. The latter may even approach or reach the temperature zone of instantaneous death.
5. The comparison between readings of soil temperatures and air temperatures show that both give useful results. For the sake of simplicity, readings of the soil temperature are preferable.
6. The importance of working with identical apparatus, in order to get comparable results, is pointed out.
7. The decrease of the modal temperature after birth is confirmed.
8. The problem of a geographical shift of the modal temperature of rodents within the same species is discussed.
9. All modal, or so-called preference, temperatures so far obtained for different species and varieties of rodents are compiled.

LITERATURE CITED

Bodenheimer, F. S. 1931. Über die Temperaturabhaengigkeiten von Insekten. III. Zeitschr. f. vergl. Physiol., 13:740–47.

Gunn, D. L. 1934. The temperature and humidity relations of the cockroach (*Blattella orientalis*). II. Zeitschr. f. vergl. Physiol., 20:617–25.

Herter, K. 1934. Biol. Zentralbl., 54:487–507.

———. 1936. Zeitschr. f. vergl. Physiol., 23:605–50.

Kalabuchov, N. 1939. Zool. Jurnal (Russian), 18:915–23.

Krumbiegel, I. 1932. Zool. Jahrb. Syst. 63:131–280.

Lütfi, M. 1936. Diss. Naturwiss. math. Fak. Berlin.

THE EFFECT OF CYANIDE ON OXYGEN CONSUMPTION OF COLPIDIUM CAMPYLUM[1]

(Two text figures)

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BEGINNING with the work of Lund (1918), oxygen consumption of ciliates has repeatedly been found to be relatively unaffected by cyanide. This is of considerable general interest, since it suggests that the mechanism by which the infusoria utilize molecular oxygen is essentially different from the usual type occurring in aerobic cells.

Loeb and Wasteneys (1913) and Warburg (1914) first demonstrated that the toxic action of cyanide on living matter is due to its specific power of inhibiting intracellular oxidations. Subsequently, it has been shown that cyanide influences the first step in a chain of reactions by which hydrogen is removed from cell metabolites and transferred to molecular oxygen. Reduction of oxygen is accompanied by oxidation of cytochrome—an intracellular hydrogen-carrier complex. This step is catalyzed by cytochrome oxidase—an enzyme which is completely inactivated in the presence of cyanide in low concentrations. Almost the entire respiration of yeast is thus inhibited. With other types of cells, notably metazoan tissue cells, it was formerly supposed that a considerable and rather variable proportion of their oxygen uptake was cyanostable and hence could not involve the cytochrome-oxidase system (Dixon and Elliott, 1929). Recently, however, van Heyningen has shown (1935) that this conclusion was based upon the results of experiments performed under improperly controlled conditions. Employing an improved technique devised by Krebs (1935), he was able to show that about 80 per cent of the oxygen consumption of tissue cells is inhibited by 0.001 M cyanide, the degree of inhibition depending directly upon the concentration of the inhibiting agent. The evidence is conclusive that the mechanism involving cytochrome and its oxidase accounts for the major portion of oxygen utilization by most aerobic cells.

In the light of van Heyningen's findings with tissue cells it seemed worth while to reinvestigate the effect of cyanide upon oxygen consumption of a species of ciliate, adopting the method devised by Krebs (1935) to eliminate the errors arising from the properties of hydrogen cyanide. The holotrich *Colpidium campylum* was chosen for the purposes of this study because bacteria-free cultures of this species were available. In testing the effect of cyanide the potassium salt was used. The addition of KCN, even in low concentrations (0.0001–0.01 M),, produces a shift in the pH of a buffered suspension of protozoa, the extent of the change depending upon the amount of KCN added, the nature of the buffer, and the initial pH. From the work of Sørensen (1909), Michaelis and Davidsohn (1911), and later investigators the sensitivity of enzyme systems to changes in H-ion concentration is now well known. It seemed advisable, therefore, as a corollary to the experiments with cyanide, to determine the relationship between oxygen consumption of *C. campylum* and the pH of the suspension.

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MATERIAL AND METHODS

Cultures of *C. campylum* were maintained, bacteria free, in 20×150-mm., cotton-plugged, Pyrex tubes containing 15 cc. of sterile medium. Transfers were made with sterile 1-ml. pipettes, observing the usual precautions in order to prevent bacterial contamination. The medium employed for cultivation of the organisms consisted of a 2 per cent solution of tryptone (Difco), containing 0.1 per cent yeast extract (Difco). The nutrient ingredients were added to a standard buffer solution containing 0.1 gm. $MgSO_4 \cdot 8H_2O$, and 0.1 gm. $Ca(NO_3)_2 \cdot 4H_2O$. The buffers employed were Sørensen's phosphate mixtures ($M/15$ KH_2PO_4-Na_2HPO_4) and Clark and Lubs's biphthalate (0.05 M $KHC_8H_4O_4$-$NaOH$). The preparation of these buffers is described in detail by Clark (1928), with data for making up solutions of known pH. The initial pH of the suspensions was determined upon a sample taken at the beginning of every experiment. In addition the final pH of the contents of the manometer vessels was measured. The pH-measurements were made with a quinhydrone electrode connected through a saturated KCl-agar bridge with a saturated KCl-calomel half-cell.

The suspensions employed in the experiments were made up from cultures freshly inoculated 48 hours before they were to be used. The cultures were kept in the laboratory except during the summer months, when the temperature became too high. During this period—from June to October—the cultures were removed to a thermostat-controlled room at 60°–65° F. The technique of preparing the experimental suspensions and of determining their density has been described in detail in a previous paper (Hall, 1938), hence it need not be repeated here.

The cyanide solutions employed in the experiments dealing with the effect of this reagent were prepared from a molar solution of KCN by diluting with fresh culture medium of the same pH as the experimental suspensions. A measured volume of a suitable dilution of 1M KCN was placed in the onset chamber of the manometer vessel and was tipped into the main chamber after a 30-minute preliminary run. The volume and concentration were adjusted so that the desired final concentration of cyanide would be approximated in the experimental suspensions.

Oxygen consumption was measured directly by means of Warburg manometers. The details of the application of the method to the present problem were stated in the earlier paper referred to above (Hall, 1938). In the current research, manometer vessels of slightly greater capacity were used, averaging about 12.5 cc. in volume. The manometers were shaken continuously at the rate of 140 cycles per minute through an arc of 5 cm. In determining the relationship between oxygen consumption and the pH of the medium, three or four manometers in addition to a thermobarometric control were used in each run, and the results were averaged. In the experiments dealing with the effect of cyanide separate controls, together with control thermobarometers, were run simultaneously with the experimental manometers and thermobarometers.

EXPERIMENTAL RESULTS

"NORMAL" OXYGEN UPTAKE

In the author's previous paper (Hall, 1938) a value of 112.5 mm.^{3} O_2/hr./10^6 was reported for the rate of oxygen consumption of *C. campylum* at 19.8° C. and pH 6.6. This figure was obtained by averaging the results of thirty-six measurements upon suspensions of ciliates in the medium in which they had been growing for 48 hours. With proper thermobarometric control the rate was found to be constant during a period of over 4 hours.

The data of these experiments provided an index of the accuracy with which samples of approximately the same density can be obtained from the original concentrated suspension using the technique described. The standard deviation of the measurements was 0.0363. For different samples from the same suspension, however, the standard deviation of the Q_{O_2}-values obtained was 0.0194. The combined error of sampling and counting, determined from the differences between counts performed on different aliquots from the same suspension, was about 4 per cent. Hence, the sampling error was well within the limits of error of the general method of relating the volume of oxygen consumed to the magnitude of the respiring population.

EFFECT OF pH

Table 1 is a summary of results of the experiments relating oxygen consumption of *C. campylum* to the pH of the medium in which the organisms were suspended. The pH given is the initial value to the nearest 0.1. Since deviations from a constant rate of oxygen uptake were slight with both of the buffers employed, the rates were calculated from the slope of the straight line which best fitted the data of each experiment. The values given in the table represent averages of the data obtained with each of the several manometers in a single run. Comparison of the results obtained in different runs performed on suspensions at the same initial pH leaves little doubt regarding the significance of the differences between rates observed at different initial pH.

The data indicate that there is a definite optimum H-ion concentration for oxygen consumption of *C. campylum* at about 2.82×10^{-6} (pH 5.55). On the alkaline side of this optimum, as the pH increases, the rate of oxygen consumption falls off gradually to about half the maximum at pH 7.65. Below pH 5.5 the rate of oxygen uptake decreases rapidly with increasing acidity.

The data in the third column of the table, indicating the magnitude and direction of the pH change which occurred in the course of each run, are particularly interesting. The greatest changes might have been expected to take place at the extremes of the pH range appropriate to the buffers employed. Inspection of the table shows that this was not the result observed. On the alkaline side of pH 5.5 the change was in the direction of increased alkalinity, and its magnitude increased as the pH; on the acid side the magnitude of the change was less, and its direction was reversed, increasing with increased acidity. The explanation of these phenomena is uncertain. In two instances with phosphate buffers (marked with an asterisk), the shift was so great in comparison with that observed in other experiments at the same initial pH that it seemed probable it was caused by creeping of the alkali in the inset chambers of the manometers into the main chambers. The results of these experiments were disregarded in subsequent analysis of the data. In all the other runs the pH change was too small to have been caused by alkali-creep. Moreover, in the suspensions buffered with biphthalate at pH below 5.5 the change was in the wrong direction to warrant such an explanation.

EFFECT OF KCN

The majority of investigators who have studied the effect of cyanide on the respiration of ciliates have found that there is an initial inhibition followed by rapid recovery and even by a period during which the respiratory rate exceeds the normal. The best studies are those of Pitts (1932) and M. Lwoff (1934). The former found that KCN in 0.01–0.001 M concentrations regularly produced a 20 per cent inhibition of oxygen consumption immediately upon introduction into a suspension of *C. campylum*. With the passage of time, however, the rate of oxygen uptake gradually increased until it surpassed the normal value. The length of time required to attain the maximum rate increased as the

TABLE 1

OXYGEN CONSUMPTION OF *C. campylum* IN RELATION TO THE pH OF THE SUSPENSION

Buffer	Initial pH	ΔpH	Q_{O_2}
Phosphate	7.5	+0.12	59.6
		+0.20	66.0
	7.4	+0.29	62.8
		+0.27	62.1
	6.8	+0.20	103.3
		+0.20	103.6
		+0.16	109.0
		+0.22	98.6
		+0.18	103.9
		+0.24	97.7
	6.4	+0.06	121.0
		+0.07	118.0
		+0.44*	106.0*
	6.3	+0.09	114.0
		+0.12	115.0
	5.5	+0.07	130.0
		+0.04	124.0
		+0.05	126.0
		+0.09	124.5
		+0.07	122.3
	5.4	+1.40*	106.5*
	5.3	+0.03	115.0
		+0.06	114.0
		+0.06	120.0
Biphthalate	5.9	0.0	125.5
		+0.02	125.5
		0.0	119.0
		0.0	123.3
		0.0	120.7
	5.4	−0.04	119.1
		−0.02	124.3
		0.0	121.6
	5.2	−0.03	86.3
		−0.03	84.5
		−0.05	86.5
		−0.04	92.0

concentration of the inhibiting agent. Lwoff secured analogous results with *Glaucoma pyriformis*. She found, however, that the initial inhibition in the presence of 0.001–0.002 M KCN might reach 80 per cent during the first half-hour.

These investigators tested the effect of cyanide by opening the manometers and adding KCN to the desired concentration after a preliminary run to establish the normal rate of oxygen consumption. Their cyanide solutions were prepared from concentrated stock solutions by "neutralizing" with a strong acid and diluting with the buffer solution in which the protozoa were suspended. The CO_2 formed in respiration was absorbed by means of a strong solution of KOH in the inset vessel of the manometers.

In the current research, experiments similar to those performed by Pitts (1932) and M. Lwoff (1934) were carried out with suspensions of *C. campylum*. The manometers were not opened during the course of the runs, however, for the purpose of introducing the cyanide, and the latter was not "neutralized" with strong acid. Typical curves for different concentrations of KCN are shown in Figure 1. The diagonal represents the average rate of oxygen consumption in the absence of cyanide, calculated from the data of the controls. The initial pH was 7.6. The black triangle on the time axis indicates the point at which the KCN solution in the onset chamber was tipped into the main chamber of the manometer vessels. The concentrations of cyanide given are approximately correct. The amount of KCN which is tipped into the suspension cannot be controlled exactly because of the fact that enough solution always remains in the onset chamber to wet the walls.

It will be observed that there is a marked initial inhibition of oxygen consumption, evident even before the KCN is tipped into the suspension. With 0.001 M KCN after 1 hour the rate of oxygen uptake surpasses that of the controls. Recovery is more gradual in the presence of higher concentrations of cyanide. In the presence of 0.005 M KCN the rate of oxygen consumption after 1 hour is about equivalent to that of the controls, while after the same interval there is still considerable inhibition with 0.01 M KCN. The data conform to the sort of curve described by Pitts (1932) and M. Lwoff (1934). This is the type which would be expected from measurements with steadily decreasing concentrations of inhibiting agent. It seems highly probable that the recovery noted after an initial inhibition of oxygen consumption is due to the disappearance of cyanide from the suspensions. The chemical properties of cyanides lend support to this conclusion.

In dilute aqueous solutions KCN reacts with water to yield KOH and HCN. The latter is a very weak acid and is extremely volatile. Hence the addition of a moderately strong acid to a solution of KCN merely serves to drive off HCN. This gas, like carbon dioxide, is readily absorbed by a strongly alkaline solution. In dilute aqueous media CO_2 dissolves to form H_2CO_3. This acid has a primary dissociation constant of 3.5×10^{-7}. The dissociation constant of HCN is 1.3×10^{-9}. In equivalent concentrations H_2CO_3 evidently will furnish approximately two hundred and seventy times as many hydrogen ions to a solution as will HCN. Thus, when cells are suspended in a buffered solution containing cyanide and are permitted to respire in a closed system (such as one of the manometers in the experiments described above), HCN will tend to distil over from the suspension into the KOH provided to absorb the respiratory CO_2. Increased glycolysis during cyanide inhibition tends to increase the rate at which the HCN distils off the suspension, since lactic acid is an even "stronger" acid ($k_a = 1.38 \times 10^{-4}$) than carbonic. As the amount of cyanide in the suspension decreases, the rate of respiration of the cells will increase; the increased CO_2-output of the cells will accelerate the disappearance of HCN until, at equilibrium, the concentration of the inhibitory agent remaining in the suspension will be negligible.

Krebs (1935) and van Heyningen (1935) have shown that the errors arising from the tendency for HCN to distil over into the CO_2-absorbing medium may be eliminated by replacing the KOH solutions usually employed for this purpose with KCN-KOH mixtures in which the concentration of free HCN is approximately the same as in the experimental fluids. When the concentration of cyanide in the suspensions is maintained reasonably constant through the use of such absorbing mixtures, respiration of *C. campylum* is found to behave like that of metazoan tissue cells. In Figure 2 are presented typical curves constructed from the data of a series of experiments performed under such conditions. Each point in the figure represents the average of the results obtained with two manometers. Again the concentrations of cyanide are approximate. The curves show

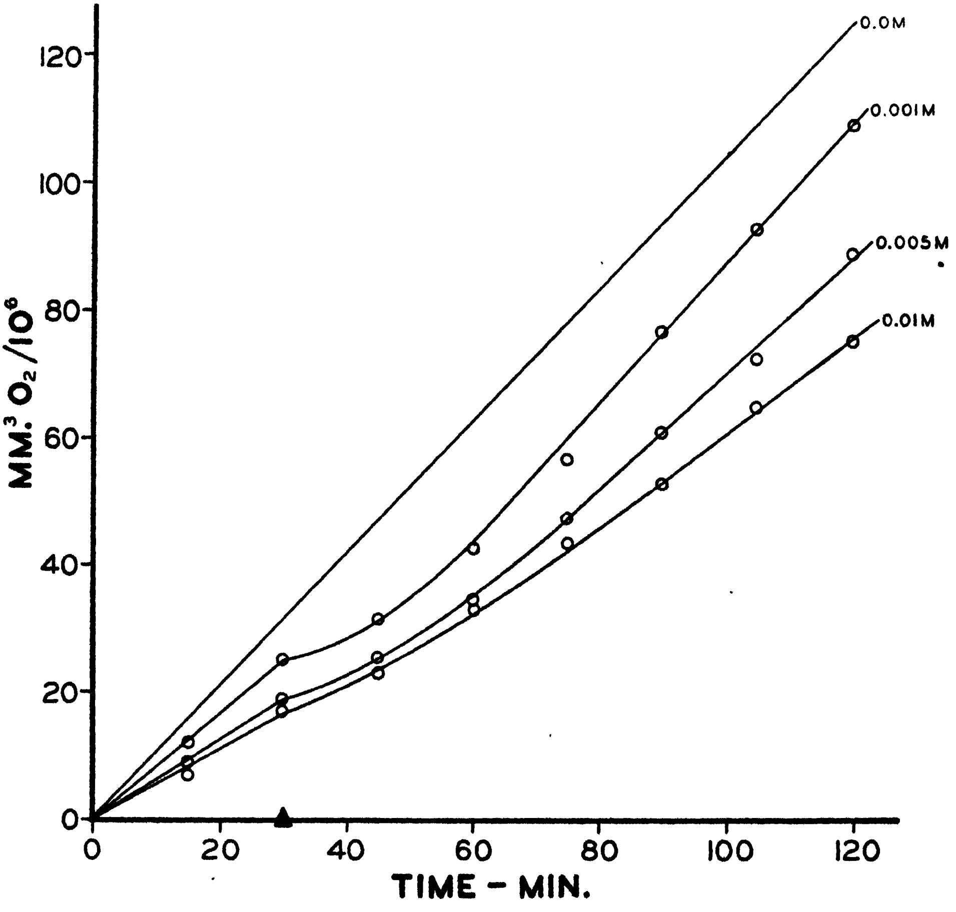

FIG. 1.—Effect of KCN on oxygen consumption of *C. campylum*. Respiratory CO_2 absorbed by KOH in inset. The figures at the right of the curves indicate the initial molar concentration of KCN.

that there is no recovery following initial inhibition of oxygen consumption. Respiration remains inhibited over a period of 90 minutes after the cyanide is tipped into the suspension. Readings were discontinued after this time. Attention is invited to the fact that, because of volatilization of HCN, the cyanide effect is present even before the KCN in

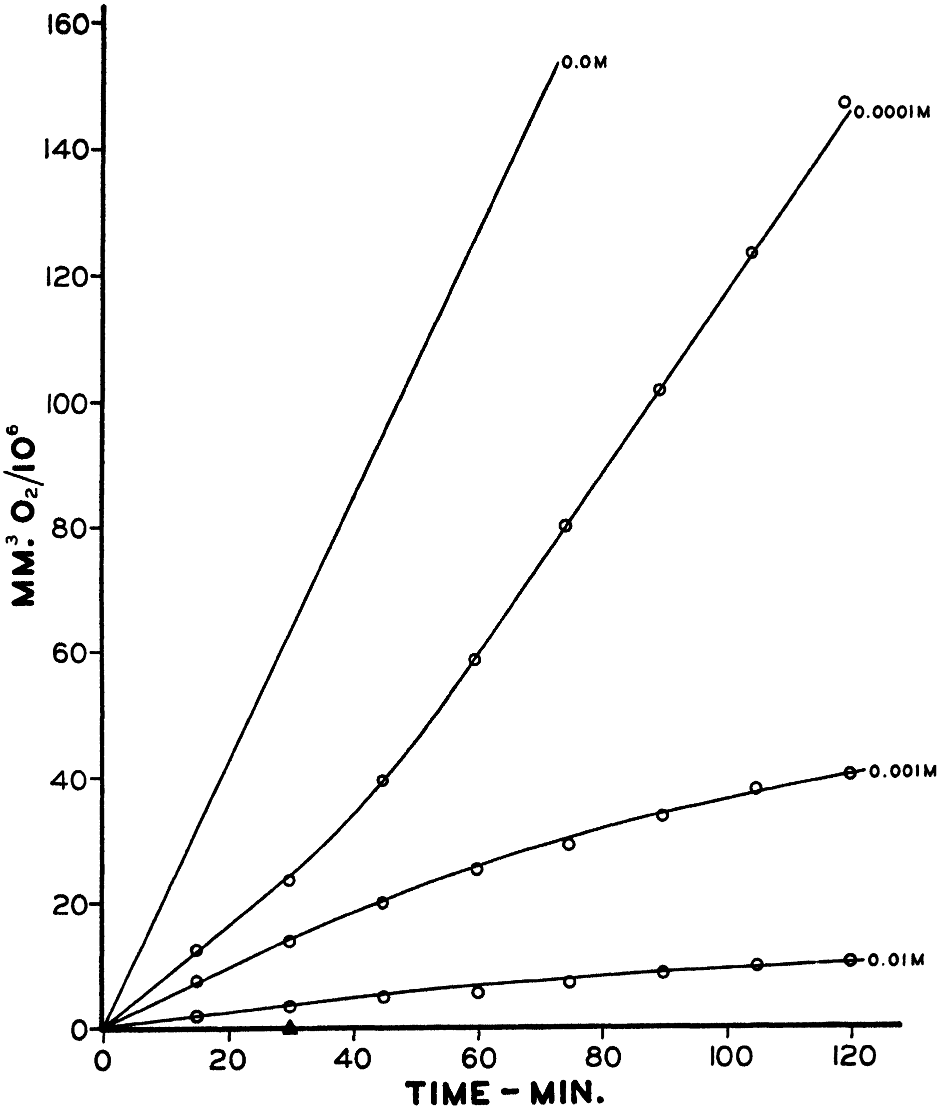

FIG. 2.—Effect of KCN on oxygen consumption of *C. campylum*. Respiratory CO_2 absorbed by means of the KCN-KOH mixtures recommended by Krebs (1935). The figures at the right, opposite the curves, refer to the initial molar concentration of KCN. The triangle on the time axis indicates the instant at which the KCN in the onset was tipped into the main chamber of the manometer vessel.

the onset chamber is tipped into the suspension. The break at 30 minutes is probably attributable in part to the shift in pH produced by the introduction of the KCN.

The extent to which respiration is inhibited is seen to depend on the concentration of cyanide. The effect is very nearly maximal with cyanide concentrations of the order of 0.001 M. The percentage inhibition with 0.001 M KCN is more than double that with 0.0001 M, while the increase with 0.01 M KCN over that with 0.001 M is very slight. The true percentage inhibition is found by comparing the rate of oxygen uptake in the experimental suspensions, 1 hour after tipping in the KCN, with the rate at corresponding pH in the absence of cyanide. The latter is obtained by interpolating along the curve fitted to the data of Table 1. The true percentage inhibition cannot be calculated by comparing the rate of oxygen consumption in the presence of a given concentration of KCN with the control for the experiment. Because of the pH change caused by the introduction of the KCN, the cells in the experimental suspensions are respiring at a different pH than the controls.

DISCUSSION

Pitts (1932) apparently was aware of the difficulty of measuring oxygen consumption in the presence of cyanide. However, in explaining the type of result which he obtained, he failed to recognize the true importance of the reversibility of the cyanide effect and the tendency for HCN to distil over into the KOH in the inset. Speaking of the recovery noted in his experiments, he states:

> This rise, following an initial inhibition, may be due in part to the volitalization of KCN, as shown by the appearance of the cyanide effect even when NaCN buffered at pH 7.4 is put in the side arm. It is extremely doubtful whether a major portion of the M/100 cyanide would have left the solution in the time involved. . . . .

From Pitts's description of the curve fitting his data there is little doubt that a major portion of the 0.01 M cyanide actually did leave the solution during the course of the experiments.

In testing the effect of cyanide on respiration, earlier investigators tried several methods of controlling exposure to the poison. Dixon and Elliott (1929) introduced the idea of neutralizing KCN in order to eliminate pH effects. In their experiments with medullated nerves Schmitt and Schmitt (cited by Robbie, Boell, and Bodine, 1938) replaced KOH as a CO_2-absorbing medium by solutions of cyanide salts sufficiently concentrated to take up the respiratory CO_2 and, because of the positive HCN-tension, also to prevent distillation of cyanide from the suspension medium. Van Heyningen (1935) was the first to employ KCN-KOH absorbing mixtures in respiration experiments. Robbie, Boell, and Bodine (1938) have checked the efficiency of these methods of control. They have shown that when no attempt is made to control exposure to cyanide, that is, when KOH is used to absorb respiratory CO_2, eggs of *Melanoplus* left in 0.0025 M KCN solutions recover their normal rate of respiration about as quickly as those removed and washed after a 3-hour exposure. Apparently, under these conditions HCN distils over into the KOH so rapidly that the cyanide concentration in the suspension medium is rather quickly reduced below the effective level for inhibition of respiration. When neutralized KCN is used, less than 30 per cent of the original amount remains in the suspension medium after 1 hour. Using a concentrated solution of KCN to absorb the respiratory CO_2, they have shown that in 3 hours the concentration of cyanide in the suspensions increases by 20 per cent of the original concentration, while in 6 hours it increases by 40 per cent. With the KCN-KOH absorbing mixtures recommended by Krebs (1935) the cyanide concentration in the suspension medium does not vary during a 6-hour period by more than 10 per cent of the original concentration.

A. Lwoff (1933) attempted to prevent loss of cyanide from his suspensions of trypanosomids by adding KCN to the KOH used in absorbing respiratory CO_2. The concentration of KCN in the absorbing mixture was adjusted so as to be equimolar with that in the experimental suspensions. M. Lwoff (1934) observed the same precaution in her experiments on *Glaucoma pyriformis*. In this connection it should be pointed out, however, that the fraction of cyanide in the absorbing mixture which would be effective in preventing loss from the suspension would be that which existed as free HCN, capable of participating in the diffusion equilibrium between both fluids and the gas space. In a strong KOH solution 0.001 M with respect to KCN, the concentration of free HCN would be very small indeed. In M. Lwoff's experiments the only effect of adding small quantities of KCN to the absorbing fluid was to prolong the recovery period. This was the result to be expected.

The causes of the difference between the results obtained by A. Lwoff (1933) for the animal flagellates and by M. Lwoff (1934) for *Glaucoma* cannot be stated at present with certainty. A possible explanation depends upon the fact that carbonic anhydrase was probably present in A. Lwoff's suspensions. A. Lwoff found that *Strigomonas fasciculata* and *Leptomonas ctenocephali* require small amounts of protohemin for growth in artificial media. The protohemin was supplied by adding fresh, defibrinated, hemolyzed dog's blood to the medium after sterilization. Carbonic anhydrase is typically present in mammalian blood (Brinkman, Margaria, Meldrum, and Roughton, 1932), from which special methods are required to separate it (Meldrum and Roughton, 1932). It seems probable, therefore, that in furnishing the necessary protohemin for the trypanosomids A. Lwoff also added carbonic anhydrase to the medium. This enzyme, which catalyzes the breakdown of H_2CO_3, is so powerful that one part in ten million is sufficient to double the rate of decomposition. By its presence, even in very small quantities, accumulation of carbonic acid in a suspension of respiring cells would tend to be prevented. Cyanide, if present, would tend to remain longer in the suspension, since the respiratory CO_2 would escape into the gas space and be absorbed by the alkali in the inset as rapidly as formed. While it is true that the enzyme is itself inhibited by cyanide, the minimum effective concentration (0.1 M, according to Meldrum and Roughton [1933]) of the poison required is much higher than for respiration. In A. Lwoff's experiments there was no recovery from cyanide poisoning because the concentration of KCN in the suspensions remained above the minimum effective level.

A similar explanation may be advanced to account for the difference between the percentage inhibition by cyanide of the respiration of ciliates and of mammalian tissue cells, where KOH alone has been used to absorb the respiratory CO_2. In preparing mammalian tissue slices for measurement of their respiration, some blood—and hence some carbonic anhydrase—must be included except when the slices are first washed. The variability of the "cyanostable" fraction of tissue-cell respiration reported by certain investigators (Dixon and Elliott, 1929) may depend upon the amount of carbonic anhydrase included in individual preparations. Using proper KCN-KOH absorbing mixtures, van Heyningen (1935) has shown that this fraction is universally small and quite constant.

There is no reason to suppose that carbonic anhydrase occurs in protozoa at all, and, indeed, in the case of *C. campylum* tests have failed to reveal its presence.[3] In the absence of carbonic anhydrase, carbonic acid would tend to accumulate in a suspension of protozoa respiring in a closed system (McBain [1912] and Thiel [1913] have shown that the breakdown of H_2CO_3 is ordinarily a relatively slow reaction). Distillation of HCN into the KOH would be accelerated by the accumulation of H_2CO_3 in the suspension medium. Consequently, respiration of the cells would tend to exhibit more rapid recovery than when carbonic anhydrase is present.

The sensitivity of respiration to poisons such as KCN, CO, and H_2S is affected by a number of factors, perhaps the most important of which is the extent of saturation of the respiratory system with carbohydrate (Warburg, 1927; Runnstrom, 1930; Keilin, 1932). A detailed discussion of these factors and their influence upon cyanide inhibition of respiration will be found in a recent paper by Commoner (1939). This investigator has shown that in concentrations of dextrose below a critical value the rate of respiration of yeast is not affected by KCN. The critical sugar concentration is lower for higher concentrations of the respiratory poison. Moreover, the maximum rate of respiration in the absence of cyanide varies as the critical dextrose concentration. Even endogenous respiration appears to be inhibited by 0.0002 M KCN. In the current research it was found that 0.0001 M KCN inhibits approximately 35 per cent of the oxygen consumption of *C. campylum* in a medium containing relatively little carbohydrate, while higher concentrations of the poison produce considerably greater inhibitions. In view of Commoner's (1939) findings these results suggest that the principal substrate oxidized by the protozoöns is carbohydrate and that the respiratory system of the cells is saturated with carbohydrate at the expense of decomposition products of the proteins in the medium.

The results of the present research, coupled with those of A. Lwoff (1933), indicate *that a relative cyanostability characterizes the respiration neither of protozoa generally nor of the ciliates in particular. The protozoa react to cyanide* in a manner similar to yeasts and tissue cells.

Since 88–95 per cent of the oxygen consumption of *Strigomonas oncopelti*, *S. fasciculata*, *Leptomonas ctenocephali*, and *C. campylum* is inhibited by 0.001 M KCN, it appears that 88–95 per cent of their respiration is dependent upon iron catalysis. A. Lwoff (1933) states that on spectroscopic examination suspensions of *S. oncopelti*, *S. fasciculata*, and *Glaucoma pyriformis* show a band at 555 mμ, which disappears when oxygen is bubbled through. In the case of *Glaucoma* the addition of $Na_2S_2O_4$ and pyridin causes the bands of the pyridin-hemochromogens of protohemin to appear. The prosthetic group of cytochrome is protohemin (Keilin), and that of cytochrome oxidase may also be (Warburg [1932] is of the opinion that this porphyrin is structurally intermediate between that of hemoglobin [protohemin] and that of leaves [phaeohemin]). From this it follows that both cytochrome and its oxidase may occur in protozoa. The effect of cyanide upon their rate of oxygen consumption suggests that the catalytic respiratory mechanism in protozoa, like that of most other aerobic cells, involves these iron compounds or closely similar ones.

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[3] The procedure described by Brinkman (1933) was employed in testing for carbonic anhydrase. Detection of the enzyme depends upon its effect on the velocity of the reaction between a 0.02 M solution of $NaHCO_3$, containing phenol red as an indicator, and a 0.006 M solution of CO_2 in water. The time required to attain the final yellow color of the indicator is taken as a measure of the reaction velocity. In the presence of carbonic anhydrase the velocity is greatly accelerated. In the current experiments fresh hemolyzed blood was employed as a standard containing carbonic anhydrase. The reaction was almost instantaneous. In the absence of any possible source of carbonic anhydrase the average reaction time was 63 seconds. With inactivated (heated for 2 minutes at 80° C.) samples of plasmolyzed ciliates, the average time was 67 seconds, while for untreated samples it was 60 seconds.

The author is unaware of any previous attempt to determine what relationship exists between oxygen consumption of protozoa and the pH of the medium. The majority of investigators heretofore either have made no effort to control the reaction of the medium in which the protozoa were suspended or have not considered this factor sufficiently important to warrant special attention. Indeed, the literature concerning the effect of H-ion concentration on oxygen consumption in general is limited, and, with the exception of the work of Tang (1936*a* and *b;* Tang and Mao-i Wu, 1938), those papers dealing with this subject which have appeared are controversial.

Warburg (1910) found that the oxygen uptake of sea-urchin eggs at pH 8.0 is nearly triple that at pH 6.0, while at pH 11.0 a further doubling occurs. The pH of the medium has been stated to be without significance as a chemical regulator of respiration of the rock barnacle, *Balanus crenatus* (Kreps, 1929) and of *Tubifex* (Dausend, 1931). In the former case, however, the measurements do not appear to have been carried out over a wide enough range to permit drawing conclusions, while in Dausend's (1931) experiments on *Tubifex* the actual magnitude of respiration was not determined, but only the frequency of movements of the hind end. Stahn (1928) found that respiratory activity of damsel-fly (*Aeschna grandis*) larvae increases as the H-ion concentration of the water from pH 7.0 to pH 6.5, then falls off with further increase in acidity. Similarly, Meyer (1935) reported a definite optimum for respiration of *Asterias* at pH 9.0. Although they do not give any supporting data, Shoup and Boykin (1931) state that for *Paramecium* "the region of change in the rate of oxygen consumption with change in pH is very narrow indeed."

*In those instances in which the effect of H-ion concentration has been studied (Nomura, 1937; Tarantino, 1937), mammalian tissues have been found to exhibit a charac*teristic optimum pH of the medium for oxygen consumption. The results of the present investigation indicate that the behavior of *C. campylum* resembles that of tissue cells in this respect. The rate of oxygen uptake is maximum in the region of pH 5.5. It is regretted that for various reasons the measurements could not be carried out over a wider range of H-ion concentrations. It was found that the medium employed for cultivation of the ciliates would not support growth at pH 5.0 or lower, either with phosphate or with biphthalate buffers. Several other buffers were tried in an effort to extend the pH range. No growth occurred at all, however, in media buffered with citrate, borate, or paranitrophenol, regardless of the H-ion concentration.

According to Tang (1936*a*) the relationship between pH and the relative rate of oxygen uptake by *Saccharomyces Wanching* in acetate buffers between pH 4.2 and pH 5.8 can be represented by the empirical equation

$$\frac{d \log A}{d\,\mathrm{pH}} = K ,$$

where

$$A = \frac{\text{rate of } O_2\text{-uptake at pH} = x}{\text{rate of } O_2\text{-uptake at pH 5.8}} .$$

Integrating, we have

$$\log A = K \times \mathrm{pH}\,.$$

If $e^K = K'$, the equation may be written

$$\mathrm{pH} = \log A - \log K'\,.$$

This is identical with the logarithmic form of the equation developed by Michaelis and Davidsohn (1911) to describe the relation between H-ion concentration and the relative activity of yeast invertase on the acid side of the optimum pH.[4]

For reasons already stated, sufficient data were not obtained at pH below 5.5 in the present research to test the applicability of Tang's (1936*a*) equation. In phthalate buffers Tang found that respiration of *S. Wanching* is relatively insensitive to pH changes between 3.6 and 6.2 but falls off steeply to zero between pH 3.6 and pH 2.6. He believes that this difference from the behavior of *S. Wanching* in acetate buffers is due to the presence within the cells of substances which combine with phthalate and also with phosphate ions to form buffering systems capable of resisting considerable change in the H-ion concentration of the medium. If we accept this explanation, it would appear that the intracellular constituents which in *S. Wanching* enter into the formation of these buffer systems with phthalate and phosphate are not present in *C. campylum*. Curiously, in regard to these latter cells, acetate in sufficient concentration to be effective as a buffer seems to be distinctly toxic.

On the alkaline side of the optimum H-ion concentration Michaelis and Davidsohn (1911) found that the pH-activity relation for yeast invertase may be roughly described by an equation of the form

$$y = \frac{cH}{k_1 + cH} \quad \text{or} \quad \frac{1}{y} = 1 + \frac{k_1}{cH}\,,$$

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[4] Michaelis' and Davidsohn's (1911) equation is:

$$y = \frac{k_2}{k_2 + cH}\,,$$

where

$$y = \frac{\text{rate of inversion at } cH = x}{\text{maximum rate of inversion}}\,,$$

and

$$k_2 = \frac{k_w}{k_b}\,,$$

$$k_b = \text{“basic” dissociation constant of enzyme}\,.$$

Writing the equation in a slightly different form, we have

$$\frac{1}{y} = 1 + \frac{cH}{k_2}\,.$$

The logarithmic form of this equation is

$$-\log y = \log cH - \log k_2\,,$$

hence

$$\mathrm{pH} = \log y - \log k_2\,.$$

where $k_1 = K_a$ = "acid" dissociation constant of enzyme, and

$$y = \frac{\text{rate of inversion at } cH = x}{\text{maximum inversion rate}},$$

$$= \text{"activity" of yeast invertase.}$$

The logarithmic form of the equation is

$$\log\left(\frac{1}{y} - 1\right) = \log k_1 - \log cH \; ;$$

hence

$$\text{pH} = \log\left(\frac{1}{y} - 1\right) - \log k_1 \, .$$

The applicability of this equation to the results of the current research has been tested by employing the average of the data obtained at a given pH-level in computing the activity coefficient y. The pertinent material is summarized in Table 2. It will be noted that the values obtained at different pH-levels for the negative logarithm of the constant k_1 (Col. 4 of the table) are in good agreement with the average, 7.65. The conclusion is permissible, therefore, that within the limits of experimental error the relationship between pH and the rate of oxygen consumption by *C. campylum* is given by the equation of Michaelis and Davidsohn (1911).

TABLE 2*

RELATION BETWEEN pH AND THE RATE OF OXYGEN CONSUMPTION OF *C. campylum*

pH	y	$\log\left(\frac{1}{y} - 1\right)$	$\text{pH} - \log\left(\frac{1}{y} - 1\right)$
5 56	0 994	$\bar{3}$ 778	7 78
5·9	0 982	$\bar{2}$ 255	7 65
6 44	0 936	$\bar{2}$ 833	7 61
6 6	0 90	$\bar{1}$ 045	7 55
7.0	0 822	$\bar{1}$.337	7 66
7.67	0.501	$\bar{1}$ 998	7.67
Average			7 65 $= -\log k_1$

* Test of the fit of Michaelis and Davidsohn's (1911) equation

$$\text{pH} = \log\left(\frac{1}{y} - 1\right) - \log k_1$$

to the averaged data of Table 1.

The data relating oxygen consumption of *C. campylum* to the pH of the medium cannot be interpreted on the basis of what is now known regarding the metabolism of this species. The results suggest that some component of the oxidative mechanism in the cells is destroyed or irreversibly inactivated below pH 5.5, while on the alkaline side of the optimum pH some component is reversibly inactivated, or its action is inhibited, by the increasing hydroxyl-ion concentration.

It has been shown that hydrolyses, dehydrations, and some oxidations and reductions catalyzed by enzymes can also be catalyzed by hydrogen and hydroxyl ions. Enzymes are generally relatively stable in the region of their optimum pH but are universally destroyed by very strong acids or alkalis. Many are inactivated in fairly weak acid or alkaline solutions, and, in general, the pH-activity curves of enzymes have been found to resemble a typical dissociation-residue curve such as Michaelis and Davidsohn (1911) obtained for invertase. These properties of enzymes have been worked out with purified preparations from cell-free extracts. The properties of enzyme systems *in vivo* are largely unknown. The possibilities for investigation along these lines with simpler systems, where the factors involved are not too numerous to be handled conveniently, have been demonstrated by Wilkes and Palmer (1932), who showed that the curve for inversion-velocity by living yeast at different pH is identical, within the limits of experimental error, with the pH-activity curve of yeast invertase.

SUMMARY

Employing the technique of Krebs (1935) and van Heyningen (1935) for measuring the oxygen consumption in the presence of cyanide, we have shown respiration of *C. campylum* to be cyanide sensitive. The percentage inhibition of oxygen consumption increases rapidly with increasing cyanide concentration up to 0.001 M, after which the increase is slight. The average inhibition with 0.001 M KCN is 88 per cent. In a medium containing relatively little carbohydrate it was found that 0.0001 M KCN inhibits approximately 35 per cent of the oxygen consumption of *C. campylum*. In view of Commoner's (1939) findings with yeast cells, this result suggests that the principal substrate oxidized by the protozoa is carbohydrate and that the respiratory system of the cells is saturated with carbohydrate at the expense of products of the decomposition of proteins in the medium.

The absence of carbonic anhydrase in ciliate protozoa and the use of KOH solutions, or KCN-KOH mixtures containing insufficient cyanide, may account for the results of previous investigators, who have reported that oxygen consumption of these cells is relatively unaffected by cyanide.

The effect of cyanide on respiration of *C. campylum* suggests that the catalytic oxidative mechanism in ciliates, like that of most other aerobic cells, involves cytochrome and cytochrome-oxidase or some system closely resembling this one.

The relationship between oxygen consumption of *C. campylum* and the pH of the suspension was investigated. The maximum rate of oxygen uptake was observed at pH 5.5. On the alkaline side of this optimum the rate gradually fell off to about one-half the maximum at pH 7.65. Within this range of pH, the data were found to fit the equation of Michaelis and Davidsohn (1911):

$$\mathrm{pH} = \log\left(\frac{1}{y} - 1\right) - \log k_1 .$$

Below pH 5.5 the rate of oxygen consumption decreased sharply with increasing acidity. The data suggest that some component of the oxidative mechanism in these cells is destroyed or irreversibly inactivated below pH 5.5, while on the alkaline side of the optimum some component is reversibly inactivated, or its action is inhibited by the increasing hydroxyl-ion concentration.

The medium employed for cultivation of the ciliates would not support growth at pH 5.0 or below, either with phosphate or with biphthalate buffers. No growth occurred in media buffered with acetate, citrate, borate, or p-nitrophenol, regardless of the pH.

LITERATURE CITED

Brinkman, R. 1933. The occurrence of carbonic anhydrase in lower marine animals. Jour. Physiol., **80**:171.

Brinkman, R.; Margaria, R.; Meldrum, N. U.; and Roughton, F. J. W. 1932. The CO_2 catalyst present in blood. Jour. Physiol. Proc., **75**:3.

Clark, W. M. 1928. The determination of hydrogen ions. Baltimore, Md.: Williams & Wilkins Co.

Commoner, B. 1939. The effect of cyanide on the respiration of baker's yeast in various concentrations of dextrose. Jour. Cell. and Comp. Physiol., **13**:121.

Dausend, K. 1931. Ueber die Atmung der Tubificiden. Zeitschr. f. vergl. Physiol., **14**:557.

Dixon, M., and Elliott, K. A. C. 1929. The effect of cyanide on the respiration of animal tissues. Biochem. Jour., **23**:812.

Fenyvessy, B. von, and Reiner, L. 1924. Untersuchungen über den respiratorische Stoffwechsel der Trypanosomen. Zeitschr. f. Hyg., **102**:109.

Hall, R. H. 1938. The oxygen-consumption of *Colpidium campylum*. Biol. Bull. **75**:395.

Heyningen, W. E. van. 1935. The inhibition of respiration by cyanide. Biochem. Jour., **29**:2036.

Keilin, D. 1932. Cytochrome and intracellular respiratory enzymes. Ergebn. d. Enzymforsch., **2**:239.

Krebs, H. A. 1935. Metabolism of amino-acids. III. Deamination of amino-acids. Biochem. Jour., **29**:1620.

Kreps, E. 1929. Untersuchungen über den respiratorischen Gaswechsel bei *Balanus crenatus* bei verschiedenem Saltzgehalt des Aussenmilieus. II. Über den Einfluss von verschiedenem pH auf den O_2-Verbrauch und über die CO_2-Abgabe bei verschiedenem Saltzgehalt des Aussenmilieus. Arch. f. d. ges. Physiol., **222**:234.

Loeb, J., and Wasten, H. yse. 1913. Is narcosis due to asphyxiation? Jour. Biol. Chem., **14**:517.

Lund, E. J. 1918. Quantitative studies on intracellular respiration. II. The rate of oxidations in *Paramecium caudatum* and its independence of the toxic action of KCN. Amer. Jour. Physiol., **45**:365.

Lwoff, A. 1933. Die Bedeutung des Blutfarbstoffes für die parasitischen Flagellaten. Zentralbl. f. Bakt., **130**:498.

Lwoff, M. 1934. Sur la respiration du cilié *Glaucoma pyriformis*. Compt. rend. Soc. biol., **115**:237.

McBain, J. W. 1912. The use of phenolphthalein as an indicator: The slow rate of neutralization of carbonic acid. Jour. Chem. Soc., **101**:814.

Meldrum, N. U., and Roughton, F. J. W. 1932. Some properties of carbonic anhydrase, the CO_2 enzyme present in blood. Jour. Physiol. Proc., **75**:15.

———. 1933. The state of carbon dioxide in the blood. *Ibid.*, **80**:143.

Meyer, H. 1935. Die Atmung von *Asterias rubens* und ihre Abhängigkeit von verschiedenen Aussenfactoren. Zool. Jahrb., Abt. allg. Zool. u. Physiol., **55**:349.

Michaelis, L., and Davidsohn, H. 1911. Die Wirkung der Wasserstoffionen auf das Invertin. Biochem. Zeitschr., **35**:386.

Nomura, K. 1937. Influence of hydrogen-ion concentration on the tissue respiration of mouse and rabbit *in vitro*, attention being paid to the buffer potency of the tissue. Tohuku Jour. Exper. Med., **31**:19.

Pitts, R. F. 1932. Effect of cyanide on respiration of the protozoan *Colpidium campylum*. Proc. Soc. Exper. Biol., **29**:542.

Robbie, W. A.; Boell, E. J.; and Bodine, J. H. 1938. A study of the mechanism of cyanide inhibition. I. Effect of concentration on the egg of *Melanoplus differentialis*. Physiol. Zoöl., **11**:54.

Runnstrom, J. 1930. Atmungsmechanismus und Entwicklungserregung bei Seeigelei. Protoplasma, **10**:106.

Shoup, C. S., and Boykin, J. T. 1931. The insensitivity of *Paramecium* to cyanide and effects of iron on respiration. Jour. Gen. Physiol., **15**:107.

Sørensen, S. P. L. 1909. Ueber die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. Biochem. Zeitschr., **21**:131.

STAHN, I. 1928. Über die Atmungsregulation, besonders die Kohlensäureregulation, bei *Dixippus morosus* und *Aeschna grandis:* Ein Beitrag zur Atmung der Insekten. Zool. Jahrb., Abt. allg. Zool. u. Physiol., **46**:1.

TANG, P. S. 1936*a*. Studies on the kinetics of cell respiration. I. The rate of oxygen consumption of *Saccharomyces Wanching* as a function of pH. Jour. Cell. and Comp. Physiol., **7**:475.

———. 1936*b*. Studies on the kinetics of cell respiration. VI. Respiration of *Chlorella pyrenoidosa* in the presence of lactates. *Ibid.*, **10**:499.

TANG, P. S., and WU, MAO-I. 1938. Studies on the kinetics of cell respiration. VII. Respiration of *Saccharomyces Wanching* in acetate, lactate, and pyruvate buffer solutions. Jour. Cell. and Comp. Physiol., **11**:495.

TARANTINO, C. 1937. La Concentrazione idrogenionica del mezzo in funzione del consumo di O_2 del tissuti. Riv. biol., **23**:281.

THIEL, A. 1913. Ueber die langsame Neutralisation der Kohlensäure. Ber. deutsch. chem. Gesellsch., **46**:241.

WARBURG, O. 1910. Ueber die Oxydationen in lebenden Zellen nach Versuchen am Seeigelei. Zeitschr. f. physiol. Chem., **66**:305.

———. 1914. Beiträge zur Physiologie der Zelle, insbesondere über die Oxydationsgeschwindigkeit in Zellen. Ergebn. d. Physiol., **14**:253.

———. 1927. Wirkung von Kohlenoxyd und Stickoxyd auf Atmung und Gärung. Biochem. Zeitschr., **189**:354.

1932. Das sauerstoffübertragende Ferment der Atmung. Zeitschr. angew. Chem., **45**:1.

WILKES, B. G., and PALMER, E. T. 1932. Similarity of the kinetics of invertase action *in vivo* and *in vitro*. II. Jour. Gen. Physiol., **16**:233.

GROWTH STUDIES ON CILIATES. V. THE ACCELERATION AND INHIBITION OF CILIATE GROWTH IN BIOLOGICALLY CONDITIONED MEDIUM[1]

(Six figures)

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EVER since the observations of Robertson (1922–27) on the growth rates of certain ciliates and their variations in response to what he called "allelocatalytic effects," there have appeared from time to time numerous papers dealing with this subject. Some of the early workers (Cutler and Crump, 1923*a*, 1923*b*, 1924, 1925) dealt directly with the problem and attempted to repeat Robertson's experiments, while others reported either support or contradiction of his hypothesis on the basis of dissimilar experiments. The general question of "allelocatalysis" has been reviewed too frequently in the past to warrant any lengthy discussion here other than to say that it is far from settled at present. Excellent discussions of the status of the problem are to be found in the works of Allee (1931, 1934), Mast and Pace (1938), and Park (1939).

It is only within the past few years that the literature has contained accounts of experiments, carried on under controlled conditions, which would throw any light upon the question as to whether the acceleration of growth or the depression of growth, often noted, could be due to the metabolic activities of the protozoa or to the associated microorganisms. With the use of bacteria-free saprozoic protozoa becoming more and more extensive one should expect this question to be revived. To date only a few papers have been published in which the problem mentioned above has been attacked under conditions permitting control of the experimental cultures by the elimination of bacteria.

Phelps (1935) in his excellent study of *Tetrahymena* (*Glaucoma*) regarding the relationship between the size of the inoculum and the growth rate found no evidence of the "Robertson effect." The growth rate and the maximum yield were the same whether he started his culture with 0.014 ciliates per ml. or 1,600 ciliates per ml. On the other hand, Mast and Pace (1938), using the flagellate *Chilomonas*, reported a significant acceleration of growth rate when many chilomonads were inoculated into a small volume of culture fluid as compared to the growth rate of single flagellate inocula. They further report that culture medium in which chilomonads had been growing for a short time possessed a substance, elaborated by the flagellates, which accelerated the growth of freshly inoculated chilomonads. This substance, according to Mast and Pace, inhibits growth, however, if its concentration becomes too great, as in the culture fluid in which a population had multiplied and died. They later (1939) reported that the substance has a molecular size not greater than 6 mμ because of its ability to diffuse through cellophane.

With these and other observations of protozoölogists, together with the many interesting reports of like phenomena among the bacteria, yeasts, and molds and even a number of the higher invertebrates as a basis, it was thought worth while to examine the problem by employing slightly different techniques. The use of bacteria-free ciliates affords one the opportunity to study cell-free conditioned medium by the simple expedient of centrifugation—a technique difficult when bacteria are being studied, especially when large quantities of medium are needed. It is, therefore, the purpose of this paper to report the results of a study of the effects of biologically conditioned medium upon the growth rate of bacteria-free ciliates of the genus *Tetrahymena* (Furgason, 1940).

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MATERIAL AND METHODS

The strain of ciliates used in this study was originally obtained from Dr. Robert H. Hall and is the one used by him for his study on oxygen consumption (Hall, 1938). It has been known in the literature as *Colpidium campylum* and was so designated in earlier reports from this laboratory (Kidder, 1939*a*, 1939*b*; Kidder and Stuart, 1939; Kidder, Lilly, and Claff, 1940) but following the investigation of Furgason (1940) it will be referred to as *Tetrahymena geleii* (Hetherington strain).

The stock ciliates were grown in 2 per cent Difco proteose-peptone–1 per cent Difco dextrose broth made up with Pyrex distilled water. This same medium was used for the bulk of the experiments here reported, although a few experiments using 1 per cent Difco yeast extract demonstrated that the general effects were the same for both.

In order to follow the growth of the populations it is necessary to make frequent counts. This involves withdrawal of volumes of medium from the growing culture; and when tubes are used the reduction soon becomes a very important factor in future growth. In the early part of the work test tubes were used, two hundred tubes of each series were inoculated, and ten tubes were sacrificed for counting purposes at each time interval; the average count was used in the records. This method was soon discarded, however, as expensive of materials and time. The bulk of the experiments were carried on in specially designed flasks.

The design of the culture flask used will be given here in detail, as it is believed that it should prove useful to future investigators of protozoan, bacterial, and yeast growth. To an ordinary Erlenmeyer flask (either 250-ml. or 1,000-ml. capacity) a side arm is fused. This side arm is of the same diameter as the mouth of the flask and is set at an angle to it. The purpose of the side arm is for inoculation in case the upper opening is used for an aeration apparatus. The aeration apparatus is placed in the flask before sterilization and need not then be removed. Near the bottom of the flask is fused a Pyrex port, shaped like the neck of a vaccine ampule, which will accommodate a rubber vaccine cap. This port is for the aseptic removal of culture medium for the purpose of counting. Before sterilization the whole port with rubber cap in place is wrapped in cotton (Fig. 1, *A*). When counts are to be made, a piece of sterile cotton is saturated with 70 per cent alcohol and substituted for the dry cotton. The flask is then agitated to insure even distribution of protozoa. With a No. 18 (2-inch) hypodermic needle, previously sterilized in a cotton-plugged serological tube (Fig. 1, *B*) and attached to a syringe (Fig. 1, *C*) before removal from the tube, the vaccine tip is punctured, the needle inserted into the body of the culture, 1 ml. or more is withdrawn, and the cotton is replaced. This sample is then delivered into a vial or tube.

By this means small volumes may be withdrawn from a single flask at frequent intervals; and, when the proper technique is mastered, the danger of contaminating the flask is practically eliminated. A single tip may be entered as many as fifty times without leaking. When 100 ml. or more of culture medium is used in a flask, the reduction of volume, even over a long period of time, is not an important factor. These flasks have proved invaluable to us, not only in the present study, but in other investigations on protozoan growth as well.

In order to test the possible presence of growth substances elaborated into the medium by the ciliates the following technique was used. Medium was prepared, and one flask was filled and set aside to act as the control on the experimental flasks. The rest of the medium was distributed into balanced, heavy-duty centrifuge tubes of 40-ml. capacity. The flask and the tubes were plugged and autoclaved. After cooling, the tubes were inoculated with stock ciliates, each tube receiving approximately the same number of ciliates (usually 100 per ml.). Growth was allowed to take place for varying periods of time, according to the experiment. This was the period of conditioning. The tubes were then all immersed in a water bath at 40° C. for 10 minutes to kill the ciliates or render them inactive. Half of the tubes were centrifuged at 3,900 r.p.m. for 6 minutes, which was sufficient to throw the ciliates to the bottom. The clear supernatant fluid of each tube was then withdrawn with a sterile 50-ml. volumetric pipette and delivered into a sterile culture flask. A fresh pipette was used for each tube, and the volume in the flask made up to 100 ml. when 250-ml. flasks were used, or to 500 ml. when liter flasks were employed. Within any single experiment all flasks were of the same size and contained the same volumes. Centrifugation (with cotton-plugged tubes) and subsequent transfers were carried out aseptically. This supernatant fluid from the ciliate culture was designated as "centrifuged-conditioned medium."

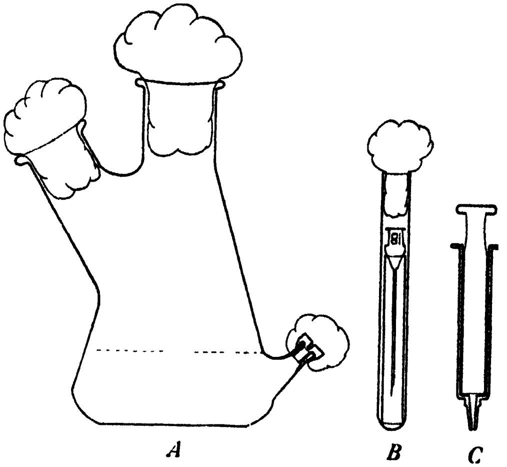

FIG. 1.—Diagram of culture flask, *A*, showing the position of the port with vaccine tip in place. The dotted line represents the height of the culture fluid. *B* shows the method used in the sterilization of hypodermic needles, which are placed on syringe *C* for use.

The fluid from the other half of the tubes was passed through sterile bacteriological filters (usually Seitz, but occasionally Berkfeld), and the sterile, ciliate-free filtrate was then transferred aseptically to flasks for future use. This filtrate was designated as "filtered-conditioned medium." Frequently, when the Seitz filter was to be employed, one operation was eliminated by sterilizing the filter in the culture flask which was to be used and then filtering directly into the flask by means of positive pressure. The flasks are too thin to allow for vacuum filtering, although the side arm can be used for suction attachment. When the requisite amount of filtrate had collected in the flask the filter was removed and a sterile cotton plug substituted.

After the two types of conditioned media had been prepared, all three flasks (unconditioned control, centrifuged-conditioned, and filtered-conditioned) were inoculated with equal volumes of the same stock ciliate culture, and the size of these initial inocula was determined for each flask by drawing samples and counting the ciliates present. Thereafter counts were made at frequent intervals (2 or 3-hour intervals during the early growth phases), and growth curves constructed from the data.

Direct counts were made of living ciliates in order that a comparison might be made between the data from these experiments and the somewhat similar data from bacterial studies, because most of the bacterial studies had utilized viable counts. Five to ten samples were always counted, and the results averaged. When dilutions were necessary as little time as possible was taken from the time the sample was drawn until the count was made, as it was thought possible that fresh broth (the diluent) might prove harmful to the ciliates from conditioned broth and tend to render the counts low.

All experimental flasks were incubated at 26° C. At the end of each experiment sterility tests both in liquid and on solid media were made, and the tests were kept two weeks for the detection of possible slowly growing contaminants. All data recorded in this report are from bacteriologically sterile cultures.

Variations from the above procedure, including the age of the inocula, will be noted and described later in the discussion.

THE NORMAL GROWTH CURVE

Several experiments were performed to determine the normal growth characteristics of *Tetrahymena* in the proteose-peptone–dextrose medium which was to be used for the conditioning investigations. The shape and slope of the curve were found to be comparable in every respect to those given by Phelps (1935) for his strain of *Tetrahymena* (*Glaucoma*). Ciliates inoculated from the logarithmic phase into fresh medium continued to reproduce at the same rate as they were doing in the previous culture. In other words, inoculum from the logarithmic phase of growth exhibited no lag phase (see control, Figs. 2, 3, 4). The numbers of ciliates present per unit volume (ml.) when plotted on a semilog scale against time give a straight line, which, when the initial inoculum is approximately 100 cells per ml., continues for from 36 to 40 hours. The slowing of the division rate is then quite abrupt, so that the curve invariably bends over sharply during the negative acceleration phase, and the stationary phase ensues. This is not at once a real stationary phase, however, as multiplication always exceeds death for many hours after the reduced rate of reproduction begins. Cultures followed for many days show the characteristic sharp logarithmic growth phase and the short negative acceleration period, followed by a slowly rising population which does not decline appreciably for weeks. For the purposes of this investigation the first part of the growth curve was selected for study, and none of the comparative experiments were continued more than 132 hours, the majority being terminated at 96 hours. Detailed data are limited to the first 72 hours in this report.

These ciliates exhibit the same general characteristics as do bacteria in that a lag phase is evidenced if the inoculum is taken from old cultures. The length of the lag phase is roughly and within limits proportional to the length of time the ciliates have spent in their old environment after the logarithmic phase of growth is passed. Ciliates taken from a 48-hour culture show very little lag, those from a 60-hour culture more, while those taken from a 96-hour culture may show very little if any multiplication for from 4 to 6 hours (see control, Fig. 5). However, the number of ciliates per ml. at the end of logarithmic growth and the maximum yield are independent of the age of the inoculum. It was decided, for the purposes of this study, that comparisons of conditioned media with control media could be made best if complications of a lag phase were omitted. Accordingly, the standard age of the inoculum for all but one set of experiments was 24 hours, or well within the logarithmic phase.

The normal growth curve (see control, Figs. 2, 3, and 4) for *Tetrahymena* (Hetherington strain) when grown in 100-ml. volumes of the proteose-peptone–dextrose medium is remarkably constant and can be reproduced at will.

TABLE 1

EFFECT OF CONDITIONED MEDIUM

MEDIUM	EXPT No.	HOURS												
		0	2	4	6	8	10	12	21	24	30	36	48	72
Control	1	100	155	200	310	480	660	980	5,900	9,600	28,500	78,000	125,000	162,000
	2	110	170	200	350	470	680	1,100	6,200	11,000	26,000	68,500	94,000	151,000
	3	100	150	210	300	480	600	960	5,700	9,800	30,000	82,500	135,000	177,000
	4	125	165	220	370	500	700	1,150	6,100	12,500	28,500	76,000	146,000	168,000
	5	100	175	190	320	490	690	1,200	5,900	13,000	27,000	91,000	130,000	173,000
	6	110	170	180	325	480	660	1,000	5,800	9,500	29,000	60,500	142,000	149,000
Av.... ..	..	107	164	200	328	480	665	1,065	5,933	12,580	28,180	76,083	128,666	163,333
Conditioned (60-hour centrifugate)	1	100	165	300	640	1,150	2,000	3,150	17,200	19,200	27,300	31,500	38,500	38,500
	2	135	190	310	750	1,600	2,600	3,950	20,500	26,100	34,200	38,000	38,000	38,500
	3	98	150	350	580	1,200	1,750	2,600	11,000	13,600	21,500	27,000	31,000	30,000
	4	100	155	280	600	990	2,150	3,900	12,000	19,000	27,500	32,500	34,500	32,500
	5	90	150	290	660	1,380	2.300	3,750	15,500	17,500	25,000	29,000	39,000	31,000
	6	100	165	300	560	1,420	2,200	3,840	19,500	21,400	19,000	26,500	36,000	35,000
Av.... .	. ..	104	162	310	631	1,290	2,166	3,531	15,950	19,466	25,750	30,750	36,166	34,250
Conditioned (60-hour filtrate)......	1	120	100	108	115	160	230	330	1,800	3,250	7,800	12,500	20,000	40,000
	2	100	110	115	120	170	260	360	2,300	3,700	8,650	14,200	26,000	36,000
	3	100	100	110	115	160	210	320	1,600	3,350	7,250	12,100	19,500	42,000
	4	85	80	90	100	120	200	290	1,200	2,600	6,550	9,900	19,500	41,500
	5	100	100	107	112	190	250	350	2,000	3,150	8,100	13,100	18,000	38,000
	6	120	110	117	121	180	240	310	2,000	3,450	7,750	12,000	16,500	43,500
Av. ..	...	104	100	107	117	163	231	326	1,816	3,250	7,683	12,300	19,916	40,166

EFFECTS OF CONDITIONED MEDIUM

When a population of ciliates has grown in a medium, they produce a change which affects the growth of a subsequent population of ciliates inoculated into the same medium. The effects are noted in (1) acceleration of division rate or (2) inhibition of growth for a period of time, depending upon the manner in which the previous population has been removed from the medium. Removal by centrifugation leaves a medium which accelerates growth; removal by filtration gives a medium which temporarily inhibits growth. The intensity of acceleration increases with the time of conditioning up to 60 hours, under the conditions of these experiments, and then decreases with further conditioning. Inhibition by the filtrate increases, within limits, directly in proportion to the time of conditioning.

MEDIUM CONDITIONED 60 HOURS

This series was repeated twelve times. The outcome of all twelve of the experiments was practically identical. Data from six experiments are presented in Table 1, and the averages are plotted in Figure 2. The six experiments presented were selected from the whole solely on the basis of time counts, all these six being counted at exactly the same time intervals. This selection was done to facilitate presentation of tabular data. Individual curves drawn from the other six are identical with the ones presented here but have been omitted for lack of space.

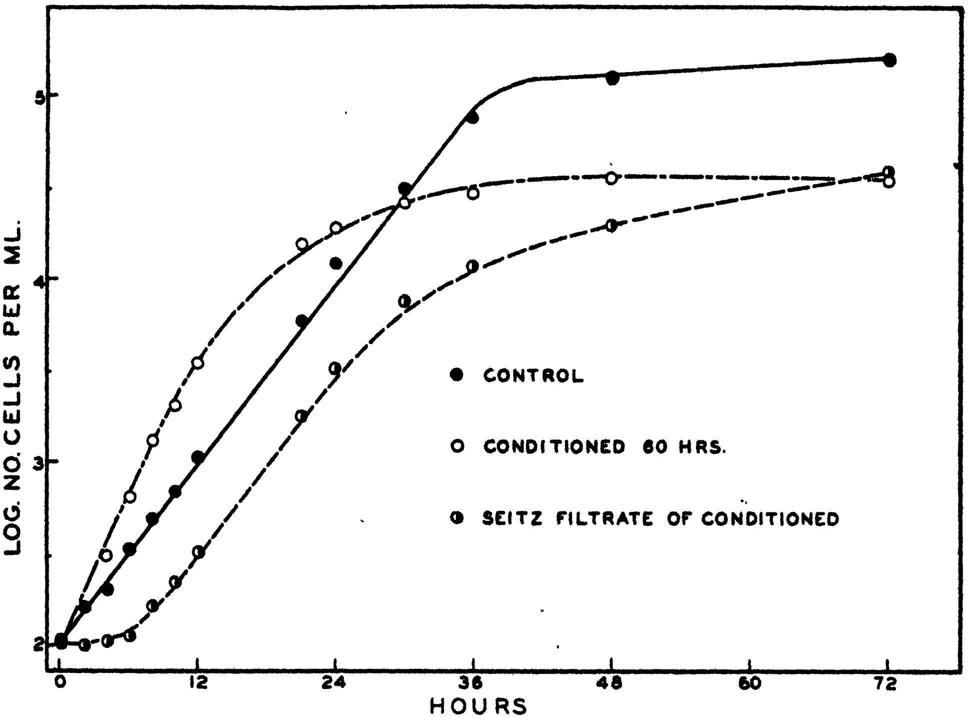

FIG. 2.—Diagrammatic representation of the data given in Table 1

It will be seen at a glance that, whereas the control cultures exhibited perfect logarithmic growth during the first 36 hours, the centrifuged-conditioned cultures show very significant acceleration over the control. This rapid reproductive rate always begins to decline early, however, and the curve flattens out very much sooner than in the control. The maximum yield is never as high as in the control. The accelerating effect is therefore evidenced only during the early growth phases, the medium proving to be below optimum for maximum population yield.

Conditioned medium from which the ciliates have been removed by either a Seitz or a Berkfeld filter is inhibitory during the early growth phases. The counts and the curves show that a pronounced lag phase appears, but the yield at 72 hours equals or slightly exceeds that of the centrifuged-conditioned medium. It was thought at first that this

TABLE 2

EFFECT OF CONDITIONED MEDIUM

MEDIUM	EXPT. No.	HOURS											
		0	2	4	6	10	12	15	24	28	36	48	72
Control........	1	130	160	260	370	730	1,500	2,000	9,000	19,000	46,000	90,000	120,000
	2	110	140	200	310	500	800	1,200	7,100	14,500	46,500	100,000	110,500
	3	150	180	300	420	680	1,200	1,700	8,500	17,000	42,500	60,000	75,000
Av.........	...	130	160	253	366	636	1,166	1,633	8,200	16,830	45,000	83,333	101,806
Conditioned (4-week centrifugate).......	1	100	170	360	570	1,140	1,500	2,200	8,500	11,600	16,400	15,500	26,000
	2	50	85	160	310	850	1,100	1,600	6,000	10,500	15,200	25,500	50,000
	3	150	240	450	810	1,600	2,400	3,950	10,500	12,500	17,500	36,500	32,000
Av.	....	100	165	323	563	1,196	1,666	2,583	8,333	11,533	16,360	25,500	36,000
Conditioned (4-week filtrate) .	1	90	80	85	100	160	200	360	1,750	2,800	6,250	13,500	39,000
	2	120	100	100	120	180	230	330	2,000	4,050	7,000	16,000	41,000
	3	110	95	100	110	170	240	290	1,200	1,950	5,500	9,000	40,000
Av....	. .	106	91	95	110	170	223	326	1,650	2,933	6,250	12,833	40,000

TABLE 3

EFFECT OF CONDITIONED MEDIUM

MEDIUM	EXPT. No.	HOURS									
		0	2	6	8	12	20	30	48	60	72
Control....	1	100	130	240	410	700	2,500	16,300	70,000	71,000	78,000
	2	80	100	210	370	650	1,900	16,000	72,000	80,000	100,000
	3	160	210	400	620	1,000	2,700	18,500	68,000	69,000	80,000
Av.	. . ..	113	146	283	466	786	2,366	16,933	70,000	73,333	86,000
Conditioned (8-week centrifugate)......	1	100	110	200	250	410	980	2,200	16,000	25,500	20,000
	2	90	100	160	210	300	760	1,800	14,000	26,000	24,000
	3	110	130	230	300	460	900	2,100	17,000	21,000	19,000
Av.........	....	100	113	196	253	390	880	2,033	15,666	24,133	21,000
Conditioned (8-week filtrate)	1	100	90	100	100	120	350	1,550	13,000	20,000	29,000
	2	100	80	90	80	110	320	1,700	10,000	26,000	36,000
	3	110	100	110	110	140	400	2,100	13,500	16,000	16,000
Av........	.....	103	90	100	96	123	356	1,783	12,166	20,666	27,000

inhibition might be due to materials added to the medium from the filter. Accordingly, fresh medium was filtered and inoculated, along with normal controls. This filtered fresh medium exhibited no inhibitory effect. The inhibition of the conditioned filtrate, then, appears to be due to the conditioning action of the ciliates. It is possible, therefore, to demonstrate both "beneficial" and "detrimental" results of the growth products of a ciliate population.

It should be noted that in these twelve experiments the growth rates for the three types of experimental media never overlapped during the first 24 hours. This means that the averages give a true representation of the actual numbers of ciliates in any one experiment at any given time.

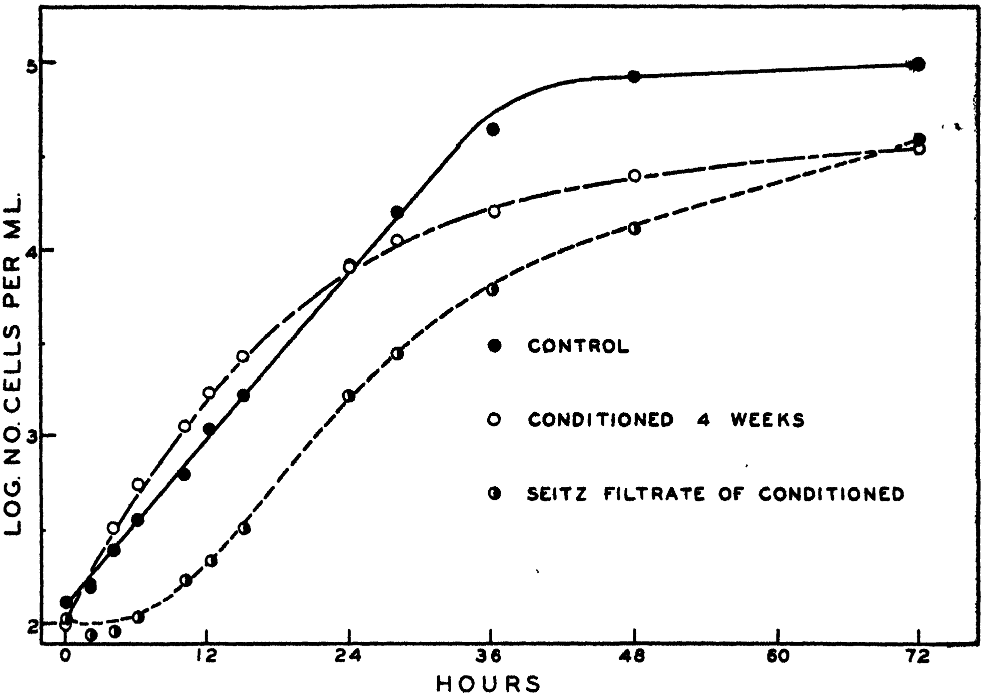

Fig. 3.—Diagrammatic representation of the data given in Table 2

MEDIUM CONDITIONED 4 AND 8 WEEKS

Inasmuch as cultures of *Tetrahymena* will remain at a high population level for many weeks, it was thought advisable to test the effect of conditioning over a long period of time. Accordingly, two series of experiments were planned, the first testing the effect of medium conditioned 4 weeks, and the second of medium conditioned 8 weeks. For these experiments liter flasks were used for the conditioning cultures. A number of flasks were set up and inoculated at varying intervals; and when the desired age was reached half the contents of a flask was transferred aseptically to centrifuge tubes, and the other half was filtered. Eight experiments were completed with the 4-week conditioned medium, and five with the 8-week conditioned medium. The results of three experiments each are given in Tables 2 and 3, and the averages are plotted graphically in Figures 3 and 4.

In medium conditioned 4 weeks and having the previous population removed by centrifugation, acceleration of growth occurred as it did in the 60-hour experiments. The acceleration was less marked, however, and the rate of reproduction decreased much sooner. The maximum yield was approximately the same as in the previous case.

The filtrate of the 4-week conditioned medium again produced inhibition, and the lag phase was somewhat longer. Again the maximum yield was about the same as that of the centrifugate.

It is apparent that conditioning up to 4 weeks renders the medium less suitable for a subsequent population than conditioning for a few (60) hours. The accelerating effect, though still significant, is diminished, while the inhibitory effect of the filtrate is more pronounced.

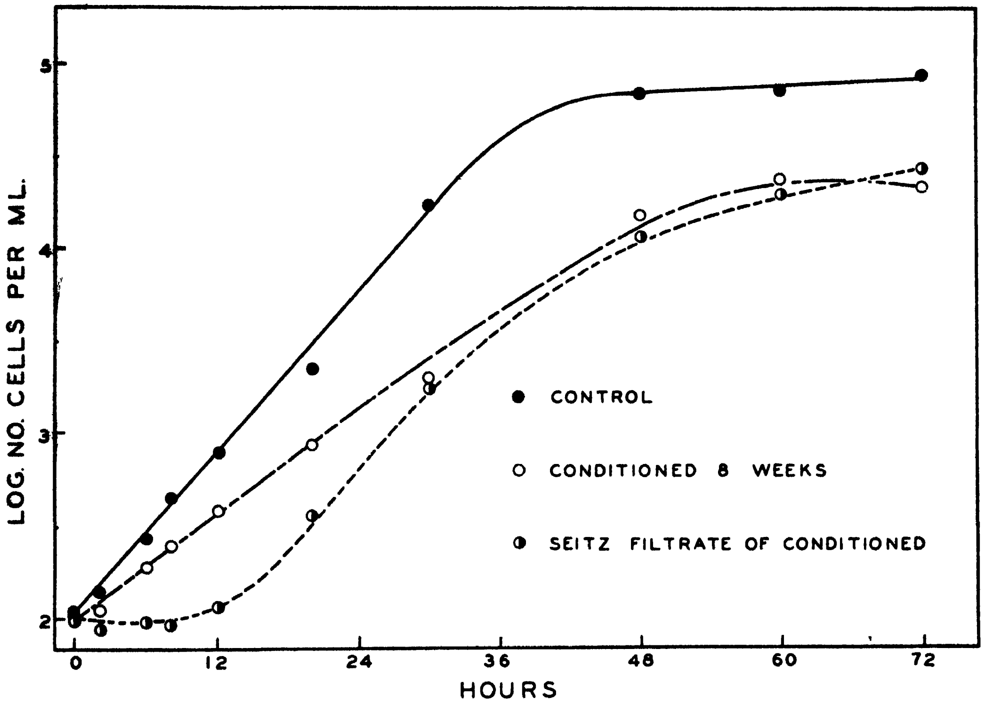

Fig. 4.—Diagrammatic representation of the data given in Table 3

After conditioning for 8 weeks the centrifugate no longer exhibited the accelerating effect as compared to the fresh medium control. The division rate was at all times less than the control, but, in comparison to the filtrate, no apparent lag phase took place.

The filtrate of the medium conditioned for 8 weeks bore the same general relation to the centrifugate during the first few hours of growth, as has been previously noted; but curiously enough the ciliates divided at a higher rate than those in the centrifugate, once the lag phase had been passed.

ADSORPTION EXPERIMENTS

Both the asbestos pads of the Seitz filter and the candle of the Berkfeld filter possess an electronegative charge. It was thought that there was a possibility that the accelerative substance, if such existed, was being adsorbed during filtration and that therefore any electronegative substance would produce the same effect even without the mechanics of filtration. Accordingly, experiments were conducted wherein animal charcoal (Norit) was used as the adsorbent. The Norit was placed in dry, 40-ml. centrifuge tubes and sterilized for 2 hours at 145° C. in a hot-air oven. After the Norit had cooled, the centrifugate from a 60-hour culture was added aseptically and mixed by shaking. The Norit was then centrifuged down, and the supernatant fluid was placed in culture flasks, 100 ml. per flask. At the same time three other types of flasks were prepared—one substituting washed plaster of Paris (electropositive) for the Norit, one with centrifuged 60-hour conditioned medium, and one with unconditioned medium. The last two served as controls. The results of these experiments are given in Table 4 and shown graphically in Figure 5.

TABLE 4

EFFECT OF ADSORPTION ON CENTRIFUGED CONDITIONED MEDIUM

(Average of two experiments)

MEDIUM	HOURS											
	0	2	4	6	8	12	16	24	30	36	48	72
Control I (untreated medium)	240	350	510	800	1,050	1,900	4,800	20,000	63,000	155,000	240,000	260,000
Control II (conditioned 60-hour centrifugate)	160	405	660	1,100	2,050	4,600	12,400	38,000	61,000	59,000	61,000	55,000
Conditioned 60 hours (adsorbed with Norit)...........	180	220	290	350	690	1,300	2,500	12,400	31,000	47,000	56,000	58,500
Conditioned 60 hours (adsorbed with plaster of Paris) ..	170	160	175	180	210	400	830	3,900	12,900	31,000	50,000	58,000

The Norit-treated medium exhibited the same degree of inhibition of growth as did the filtrate in the previous 60-hour experiment. The lag phase was of about the same duration, and the general shape and height of the growth curve was the same.

The medium treated with plaster of Paris yielded somewhat surprising results. Not only was there inhibition of growth, but the lag phase was extremely long, and it was not until after 12 hours that the division rate was as high as in the Norit-treated medium.

These experiments were repeated with the same results. The inhibiting effect could not be due entirely to the adsorption of some accelerating substance unless the substance is capable of shifting the sign of its electrical charge, because both electronegative and electropositive materials acted in the same manner. Separate experiments, wherein plain, unconditioned medium was treated as above with both Norit and plaster of Paris, yielded growth curves similar to the untreated controls. No materials essential for maximum growth under these conditions appear to be removed from normal medium, nor do detrimental substances appear to be added.

TEMPERATURE AND TIME

The effects of boiling temperatures on the 60-hour conditioned medium after the cells had been removed was tested. Both the accelerative effect of a centrifugate and the inhibiting effect of a filtrate are completely destroyed at 100° C. (Table 5). Boiled centrifugate and filtrate lose their conditioned effect and produce curves comparable to the normal control curves but with a lower maximum yield.

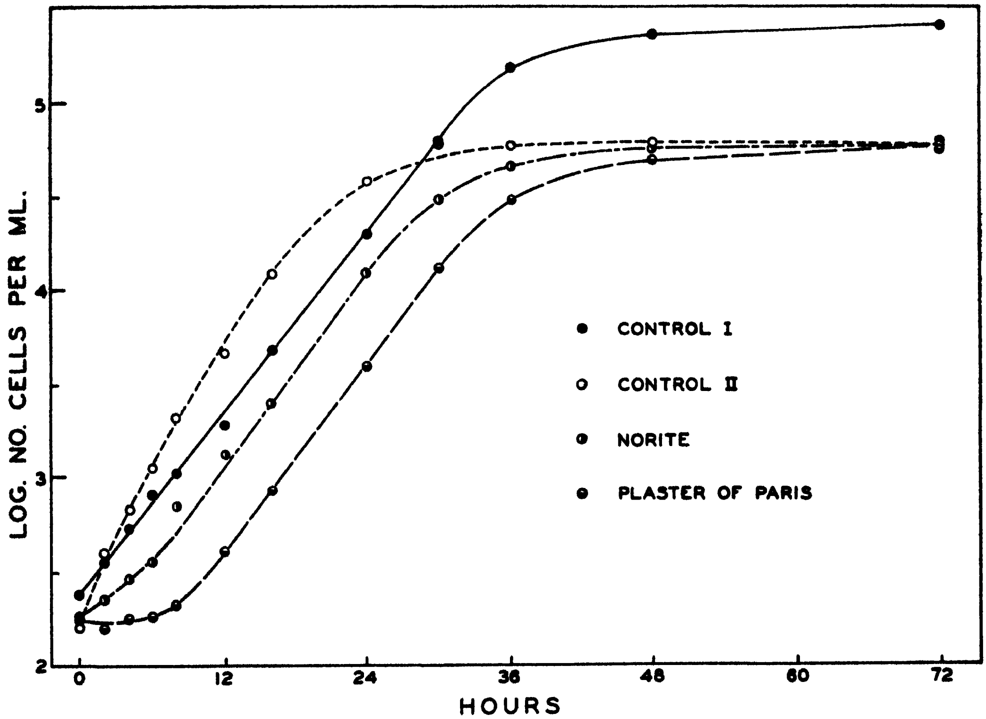

Fig. 5.—Diagrammatic representation of the data given in Table 4

If 60-hour conditioned medium is handled as described in the first set of experiments (Table 1) but allowed to stand uninoculated for 72 hours after the previous population has been removed, both the accelerating effect of the centrifugate and the inhibiting effect of the filtrate diminish but do not completely disappear (Table 6). Conditioning effects do disappear after 10 days, however.

ACCELERATION AND THE LAG PHASE

As was previously stated, no lag phase appears if ciliates from the logarithmic phase of growth are inoculated into untreated proteose-peptone–dextrose medium. When ciliates from an old culture are used for inoculation, however, a lag phase[2] appears. What effect will the accelerating substance of centrifuged conditioned medium have on old ciliates immediately after inoculation? To answer this question a centrifugate of medium conditioned 60 hours, together with a control of untreated medium, was inoculated with ciliates from a 7-day-old culture. This experiment was repeated three times, and the data from the four experiments are given in Table 7 and shown graphically in Figure 6.

The general relationship of 7-day inoculum in the centrifuged, 60-hour medium to 7-day inoculum in the control is the same as when young ciliates (taken from the logarithmic phase) are used. The ciliates exhibit only a slight indication of a lag phase in the former medium and an appreciable lag in the control. This is a very interesting point in that it has long been accepted that, in bacterial and yeast growth, a lag phase is the inevitable outcome of old inocula.

TABLE 5

EFFECT OF HEATING OF CONDITIONED MEDIUM

(100° C. for 5 minutes; average of four experiments)

MEDIUM	HOURS									
	0	2	6	10	12	24	36	48	60	72
Control	90	124	250	520	790	7,700	66,000	102,000	110,000	125,000
Conditioned (60-hr. centrifugate)	75	90	185	410	600	5,200	15,200	31,000	46,000	70,000
Conditioned (60-hr. filtrate)....	103	155	330	720	1,050	8,000	17,000	25,000	39,000	51,000

TABLE 6

EFFECT OF AGING OF CONDITIONED MEDIUM

(Inoculated 72 hours after removal of previous population; average of two experiments)

MEDIUM	HOURS								
	0	4	6	10	12	24	36	60	72
Control....................	120	200	330	610	900	8,600	31,000	78,000	100,000
Conditioned (60-hr. centrifugate)	100	260	490	920	1,400	8,800	16,000	29,000	36,000
Conditioned (60-hr. filtrate)...	100	105	125	300	410	3,500	14,000	31,000	46,000

If one thinks of the lag phase as being a period of adjustment or acclimatization to a new environment, then the above result might be expected. The medium which has been conditioned for 60 hours is more nearly like the medium from which the inoculum was taken than is fresh broth. At any rate this seems to indicate that the cause for a lag phase, which may be due to the previous environment of the inoculated organisms, may be modified considerably by the choice of media one may use.

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[2] The term "lag phase" as used in this discussion is the period elapsing between the inoculation and the beginning of logarithmic growth. No attempt has been made—and the data does not warrant such an attempt—to break this phase up into the "initial stationary phase" and the "lag phase" of Buchanan (1918).

DISCUSSION

The data which have been accumulated with the use of the techniques described above allow one to put forth some tentative interpretations regarding their meaning. It is possible to obtain duplications of the results of any of the sets of experiments under these conditions, and one might be tempted to generalize as to their meaning. As a matter of fact, the general phenomena reported here for *T. geleii*(Hetherington strain) apply equally well to at least two other strains of the same species (Elliott strain and Phelps strain), as I have previously reported (Kidder, 1939*b*). Whether these results will be obtained when still other species and genera are employed is, of course, impossible to say.

TABLE 7

EFFECT OF CONDITIONED MEDIUM ON THE LAG PHASE

(Age of inoculum, 7 days; average of four experiments)

MEDIUM	HOURS									
	0	2	4	6	12	14	24	36	48	72
Control .	100	100	105	130	350	640	2,600	21,000	85,000	126,000
Conditioned (60-hr. centrifugate). . . .	100	125	160	240	710	1,600	6,300	15,500	23,000	30,000

Mast and Pace (1938), after a long series of experiments, concluded that *Chilomonas paramecium* elaborated a substance (designated as *X*), which in low concentrations accelerates the growth of freshly inoculated chilomonads "from a vigorous culture" (p. 361) (optimum 1 part in 10,000,000) but inhibits growth or causes death when the concentration becomes high. They claim to have demonstrated the "Robertson effect," in that the rate of reproduction was higher when many chilomonads (up to 50) were inoculated into a given small volume (0.4 cc.) than when few (1 or more) were used. Furthermore, they show that as the volume of culture fluid is decreased the rate of reproduction of a single chilomonad rises. They believe that the elaborated substance is necessary for reproduction and that when the substance is low in the medium a certain amount of time is needed for its production. They, therefore, confirm the original hypothesis of Robertson under considerably more controlled conditions. They state that substance *X* is heat labile, being destroyed in proportion to the time of heating (100° C.). These conclusions are of great significance.

The accelerative effect which was demonstrated in the present study seems to compare favorably with the work on *Chilomonas*. It would be necessary only to assume that the so-called *X*-substance is elaborated more slowly in *Tetrahymena* and that it does not act in such high dilutions. Moreover, it has not been shown to be lethal in high concentrations, although it does lose its accelerative powers with age. Perhaps, if both the

Tetrahymena and the *Chilomonas* materials had been handled in the same manner, more points of similarity would have been noted.

It does not seem probable that a comparison can be made between the inhibitory effect of the conditioned filtrate and the inhibition noted by Mast and Pace, because of differences inherent in the techniques employed. Although, if one is to look upon the inhibitory effect of the filtrate as being due to elaborated substances, then it may be that the old *Tetrahymena* culture medium contains more and more of this substance, thereby masking the effect of a possible accelerative substance. The experiments of Mast and Pace, wherein they heated their *X*-containing medium for varying periods of time, seem to rule out direct comparison, however.

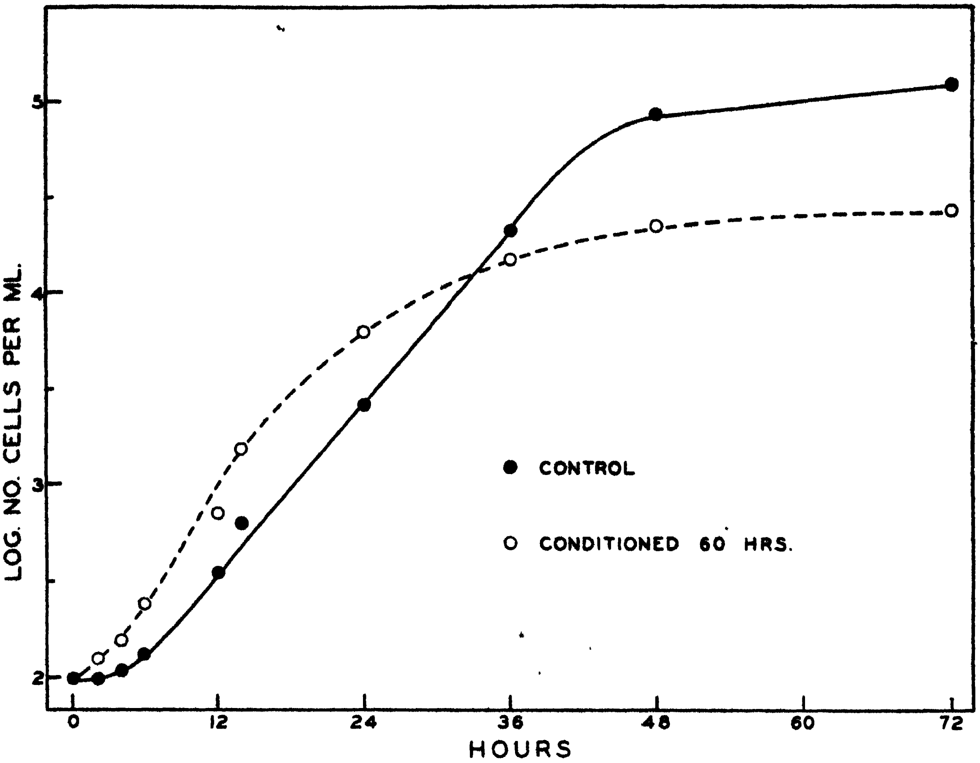

FIG. 6.—Diagrammatic representation of the data given in Table 7

A possible explanation of the phenomena reported here may be tentatively expressed as follows. In a growing culture of *Tetrahymena* two types of substances are being elaborated by the cells—one an accelerator which is produced at a high rate during the first few hours (up to 60) of growth, the other type detrimental and inhibiting to growth. Both types of substances are heat labile so that heat-treated conditioned medium is restored to the level of fresh broth (as far as early growth rate is concerned). The accelerator appears to dominate the activity of the growing cells because it exerts its effect in the presence of the inhibitor as in the centrifugate. Removal of the accelerative substance or substances seems to be brought about by filtration or adsorption, thereby allowing the inhibitor to become effective. It is evident that the accelerative substances are not removed by the filter because of their size (mechanical filtration) but by adsorption (see the experiment with Norit), but it is also indicated that they may be able to change their electrical charge as they appear to be adsorbed on electropositive plaster of Paris.

Growth inhibition due to filtrates from old cultures seems to be the rule in bacterial studies. Inhibitory substances have been shown to pass through a collodion membrane (Wheeler and Stuart, 1937) and collect in a sterile broth which was separated from a growing culture. There is the possibility that, could the previous population of a bacterial culture be removed by centrifugation rather than by filtration, then acceleration would be the result.

A more complete understanding of the substances involved during biological conditioning must await further investigation. Carbon dioxide tension, which is known at times to accelerate in high dilutions and depress in higher concentrations, might be a factor in the present study. There is some evidence against this interpretation, however. Untreated medium has a pH of 6.8. Medium conditioned for 60 hours has a pH of 5.8–6.0. This drop in pH value occurs only in the presence of the sugar, as plain proteose-peptone medium grows slightly alkaline with the advancing age of the culture. When conditioned medium is passed through a Seitz filter, the first 10–15 ml. collected has a pH of 6.8, showing that the acid-producing material is being taken out. This might be carbon dioxide as filtration brings about rather complete aeration. But subsequent collections from the filter show that the pH of the filtrate is dropping. After 100 or more ml. have passed through, the value is very near that of the centrifugate of the conditioned medium. This indicates that the filter is actually adsorbing the acid-forming substance and that eventually the filter pad becomes saturated. It is difficult to imagine the acid-forming substance as being CO_2, for aeration is just as complete at the end of a filtration as at the beginning. Of course, the shift in pH value during filtration may be due to the addition of some basic substance from the filter, but in either case the importance of CO_2 is minimized.

Recently Hall and Loefer (1940) reported the effects which they obtained by the use of old, uninoculated medium and old culture filtrate. Because of the rather obvious connection between their claims and the data reported here (in fact, the statement is made that I confirm the results given in their earlier abstract [Hall and Loefer, 1938]) it will be necessary to analyze their reports in some detail. The organism they used for their experiments is called by them *Colpidium campylum* but is the same strain of *Tetrahymena* which I used (Hetherington strain, see above).

Because of the method of handling their material I do not agree that my data (reported in abstract form [Kidder, 1939*b*]) confirms their results or their interpretations. The following points will suffice, I believe, to show that the conditions under which they worked could not have been expected to bring out data which could be used for comparisons with previous work or the data reported here.

They claim to have obtained evidence that the addition of a filtrate (Whatman No. 12 filter paper!) of an old culture (7 weeks–3 months) or the addition of sterile aged (3 months) medium to fresh medium increased both the rapidity of growth and the yield of their ciliates. Their basic medium was a casein peptone (Difco tryptone), and the efficacy of this medium can be judged by the control of their first experiment (Series II), in which, after 144 hours with an initial inoculum of 67 ciliates per ml., the yield was only 1,327 ciliates per ml. Compare this to the controls (unconditioned medium) given above when a more adequate medium is used (100,000–125,000 per ml. at 72 hours). Furthermore, there is a number of discrepancies in their report. In the first set of experiments their inoculum was from a 48-hour stock culture, and each tube received 1.0 cc. volumes of the inoculum. This inoculum resulted in an initial concentration of 67 per cc. In their second and third experiments each tube received only 0.5 cc. from a 48-hour culture, and yet they give the initial concentration of all tubes in these two experiments as 1,219 ciliates per cc. One can only conclude that they are getting remarkable inconsistencies in growth in their stock cultures. The control in the first experiment yielded only 1,327 ciliates per cc. at the end of 144 hours, which they consider as better than their Series V and VIII (controls), because the increase in the first case was twenty times the inoculum at the end of 6 days, while in the others it was less than eight times the inoculum. It is difficult to reconcile this statement with the easily demonstrated fact that no matter what the number of cells in the inoculum, provided it is less than the number reached at the end of logarithmic growth and provided the inoculated cells live, the yield is the same under the same conditions of medium and temperature. We have always been able to verify this fact; and it was perfectly demonstrated in the work of Phelps (1935), as it has been in innumerable cases with bacteria and yeasts.

Hall and Loefer were not using what I refer to here as biologically conditioned medium, because after aging, either in the presence or in the absence of ciliates, they subjected their experimental fluids to the temperatures and pressures of the autoclave rather than removing the previous population aseptically.

The demonstration of both acceleration of growth and inhibition of growth from the medium which has supported a previous population would seem to leave the way open for a revaluation of some of the contradictions in the literature regarding the actuality of the "Robertson effect." This is made difficult, however, by the fact that very few of the investigations dealing with this question were carried on under controlled conditions. We have no notion as to the role of the associated microörganisms in the reports; so it appears that, if this interesting question is to be settled, the early work will have to be repeated on sterile organisms. Even then the *modus operandi* will not be understood until we are in a position to make biochemical analyses of what may possibly prove to be elaborated enzymes or even more delicately arranged chemical materials. At present we can only point to the results and admit that the explanations are conjectures.

SUMMARY

1. When sterile *T. geleii* are grown in proteose-peptone–dextrose broth, they exhibit reproducible growth characteristics.

2. Young ciliates inoculated into fresh broth exhibit no lag phase but reproduce logarithmically until the negative acceleration phase is reached, when the division rate decreases.

3. Old ciliates inoculated into fresh broth exhibit a lag phase, but the maximum yield is the same as when the inoculum is young.

4. Medium which has supported a population of ciliates for up to 60 hours possesses the power either to accelerate or to inhibit the growth of a subsequent population. If the previous population is removed by centrifugation, acceleration results. If the pre-

vious population is removed by passing the fluid through a bacteriological filter (Seitz of Berkfeld), inhibition results.

5. The accelerating factor or substance decreases with the age of the previous culture after 60 hours, so that medium conditioned 4 weeks shows only slight but significant acceleration while medium conditioned 8 weeks shows no acceleration (as compared to fresh broth).

6. Medium conditioned 4 and 8 weeks and then filtered shows progressively more inhibition.

7. Centrifuged, 60-hour conditioned medium when treated with sterile charcoal (Norit) or plaster of Paris loses its accelerative ability and produces inhibition of growth.

8. All conditioning effects, except the lower maximum yield, are destroyed by heat and also lose their activity upon standing.

9. Centrifuged, 60-hour conditioned medium when inoculated with old ciliates diminishes or obliterates the lag phase.

10. It is suggested that two substances may be elaborated by the growing ciliates, one an accelerator which acts freely during the early growth phases but which is removed either by filtration or by adsorption. The other substance produces inhibition of growth, and when the accelerator is removed its full effect is noted.

LITERATURE CITED

ALLEE, W. C. 1931. Animal aggregations. Chicago: University of Chicago Press.

———. 1934. Recent studies in mass physiology. Biol. Rev., **9**:1–48.

BUCHANAN, R. E. 1918. Life phases in a bacterial culture. Jour. Infect. Dis., **23**:109–25.

CUTLER, D. W., and CRUMP, L. M. 1923*a*. The rate of reproduction in artificial cultures of *Colpidium colpoda*. I. Biochem. Jour., **17**:174–86.

———. 1923*b*. The rate of reproduction in artificial cultures of *Colpidium colpoda*. II. *Ibid.*, pp. 875–86.

———. 1924. The rate of reproduction in artificial cultures of *Colpidium colpoda*. III. *Ibid.*, **18**:903–12.

———. 1925. The influence of washing on the reproductive rate of *Colpidium colpoda*. II. *Ibid.*, **19**: 450–53.

FURGASON, W. H. 1940. The significant cytostomal pattern of the "*Glaucoma-Colpidium* group" and a proposed new genus and species, *Tetrahymena gelii*. Arch. f. Protistenk. Vol. **94**.

HALL, R. H. 1938. The oxygen-consumption of *Colpidium campylum*. Biol. Bull., **75**:395–408.

HALL, R. P., and LOEFER, J. B. 1938. Effect of the addition of old culture medium on the growth of *Colpidium campylum*. Anat. Rec., **72**:50 (abstract).

———. 1940. Effects of culture filtrates and old medium on growth of the ciliate *Colpidium campylum*. Proc. Soc. Exper. Biol. and Med., **43**:128–33.

KIDDER, G. W. 1939*a*. The effect of biologically conditioned medium on the growth of *Colpidium campylum*. Biol. Bull., **77**:297–98 (abstract).

———. 1939*b*. The effects of biologically conditioned medium on the growth rate and population yield of certain ciliated protozoa. Science, **90**:405–6 (abstract).

KIDDER, G. W.; LILLY, D. M.; and CLAFF, C. L. 1940. Growth studies on ciliates. IV. The influences of food on the structure and growth of *Glaucoma vorax* sp. nov. Biol. Bull., **78**:9–23.

KIDDER, G. W., and STUART, C. A. 1939. Growth studies on ciliates. I. The role of bacteria in the growth and reproduction of Colpoda. Physiol. Zoöl., **12**:329–40.

MAST, S. O., and PACE, D. M. 1938. The effect of substances produced by *Chilomonas paramecium* on rate of reproduction. Physiol. Zoöl., **11**:359–82.

———. 1939. The size of the molecules in the growth substance produced by *Chilomonas paramecium*. Anat. Rec. Suppl., **75**:77 (abstract).

PARK, T. 1939. Analytical population studies in relation to general ecology. Amer. Midland Naturalist, **21**:235–53.

PHELPS, A. 1935. Growth of protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and the amount of the inoculum. Jour. Exper. Zoöl., **70**:109–30.

ROBERTSON, T. B. 1921*a*. Experimental studies on cellular multiplication. I. The multiplication of isolated Infusoria. Biochem. Jour., **15**:595–611.

———. 1921*b*. Experimental studies on cellular multiplication. II. The influence of mutual contiguity upon reproductive rate in Infusoria. *Ibid.*, **15**:612–19.

———. 1922. Reproduction in cell communities. Jour. Physiol., **56**:404–12.

———. 1924*a*. The nature of the factors which determine the duration of the period of lag in cultures of Infusoria. Australian Jour. Exper. Biol. and Med. Sci., **1**:105–20.

———. 1924*b*. The influence of washing upon the multiplication of isolated Infusoria and upon the allelocatalytic effect in cultures initially containing two Infusoria. *Ibid.*, pp. 151–75.

———. 1924*c*. Allelocatalytic effect in cultures of *Colpidium* in hay infusion and in synthetic medium. Biochem. Jour., **18**:1240–47.

———. 1927. On some conditions affecting the viability of Infusoria and the occurrence of allelocatalysis therein. Australian Jour. Exper. Biol. and Med. Sci., **4**:1–24.

WHEELER, K., and STUART, C. A. 1937. Growth inhibition of *Echerichia coli*. Jour. Bact., **33**:525–31.

A FURTHER STUDY OF ENVIRONMENTAL FACTORS AFFECTING CYSTMENT IN WOODRUFFIA METABOLICA

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NORMALLY, *Woodruffia metabolica* forms resting or protective cysts in the absence of food (Johnson and Evans, 1939). Depending upon environmental conditions at the time of encystment, this ciliate will form one of two types of protective cysts, which are distinguishable both physiologically and morphologically (Johnson and Evans, 1940). We refer to them as "stable" and "unstable" cysts. Excystment of stable cysts occurs only upon the addition of some plant extract, while the excystment of unstable cysts can be induced by the mere addition of distilled water or by the abrupt lowering of the temperature. Morphologically, the unstable cysts are distinguished from the stable type by the possession of fluid vacuoles, larger protoplast, more diffuse cytoplasmic granules, less viscosity of the protoplasm, and generally a thinner ectocyst and mesocyst.

Other investigators have reported cysts of protozoa which can be induced to excyst without the addition of any organic substance. For example, Moore (1924) has reported the spontaneous excystment of *Spathidium*. Darby (1929) was able to cause excystment of *Stylonychia* by addition of fresh water to the cysts. Garnjobst (1937) induced excystment of *Euplotes taylori* and *Stylonethes sterkii* upon the addition of tap or distilled water. Kidder and Stuart (1939) regularly brought about excystment of *Colpoda* simply by adding distilled water. Taylor and Strickland (1938) have observed the spontaneous excystment of *C. duodenaria* under certain conditions in sterile inorganic medium. *Woodruffia*, which have been induced to encyst and excyst three or four successive times without feeding, have been seen to excyst spontaneously in inorganic medium upon numerous occasions; the sensitivity of these small cysts to slight changes of the environment is apparently intensified. A comparative study of the different protozoan cysts referred to above should be carried out in the same laboratory.

The occurrence of unstable cysts in *Woodruffia* was discovered quite by accident. During some tests in which encysting organisms were subjected to higher temperatures, we were surprised when the mature cysts in control dishes excysted merely with the addition of distilled water. It was also striking that the excystment time was only 50–75 minutes at room temperature, whereas the excystment time of stable cysts is 12–15 hours under the same conditions. Experiments were then devised to find the factors which caused the formation of unstable cysts, the results of which have already been reported (Johnson and Evans, 1940). In that study it was found that various factors such as high temperature, excessive crowding, starvation of food organisms, and moderately increased salt concentration of the medium were effectual to a small degree in stimulating the formation of unstable cysts. When these factors were combined, the stimulating effect was considerably increased. It was also found that a total salt concentration of 1.2 per cent (Osterhout's balanced salt one hundred times that generally used) would induce nearly 100 per cent formation of unstable cysts.

The purpose of the present investigation was to extend these studies on factors concerned in the formation of unstable cysts and to determine if possible whether several factors acting independently operate in producing the unstable cyst or whether there is some underlying single factor which causes the formation of such cysts.

MATERIALS AND METHODS

The *Woodruffia* used for this investigation were derived from one of our stock cultures. The methods used in maintaining stock cultures have been described in detail elsewhere (Johnson and Evans, 1940). *Paramecium multimicronucleata* was used as the food organism in both the stock cultures and the cultures of *Woodruffia* which were used as experimental animals.

A number of carefully washed paramecia were transferred to isolation cultures each containing a suspension of *Pseudomonas fluorescens*. The progeny of the culture exhibiting the highest reproductive rate were distributed among four 250-cc. Erlenmeyer flasks each containing balanced salt solution. To each was added three standard loops of *P. fluorescens* daily. These flask cultures were kept at 26° C. After 2–3 weeks the maximum population of paramecia was reached (450–500 per cubic centimeter); until this time none was used as food for *Woodruffia*. The paramecia of such cultures were similar in size and appearance. Whenever paramecia were pipetted out for *Woodruffia*, fresh balanced salt was added to the flask to maintain the original volume.

The bacteria were obtained from the Stanford department of bacteriology from the strain designated "U6" and grown on Difco nutrient agar. The bacteria used as food for the paramecia were always taken from 4-day-old plates.

Hundreds of paramecia were prepared for use daily by washing twice in balanced salt solution by centrifuging. These paramecia were transferred to Columbia watch dishes, to each of which was then added a few *Woodruffia*. An adequate source of experimental animals was thus available at any time.

As long as food is present, most of the *Woodruffia* are either engaged in division or have formed temporary or digestion cysts. The latter type resembles division cysts, but no division occurs. When the food has been depleted in a culture, all the *Woodruffia* become free swimming almost simultaneously for a short time. At this cultural phase, experimental animals were removed and washed, preparatory to encystment.

Balanced salt solution was prepared with triple Pyrex-glass–distilled water and C.P. salts (Merk.), and buffered at a pH of 6.9–7.0. Johnson (1933) has described the method for preparing the salt solution. The solution was filtered through a porcelain filter before use. *Woodruffia* to be used for experiments were always washed twice in the balanced salt solution by transferring with clean pipettes.

Columbia watch glasses were used as experimental dishes. Before use these dishes were cleaned thoroughly by scouring, then rinsed in distilled water, and dried with a clean towel. Experiments at 10° C. were carried out in a constant-temperature room with a fluctuation of not more than 0.2° C. Experiments at all higher temperatures were conducted in box incubators with a fluctuation of not more than 0.4° C. Petri dishes (diameter, 10 cm.) were used as moist chambers. At higher temperatures (30–37° C.) the Petri dishes were placed in large moist chambers for added insurance against evaporation.

In the following experiments *Woodruffia* experimental cultures were prepared in two ways. The method most generally used consisted in transferring 30 washed *Woodruffia* to an experimental dish containing 1 cc. of medium. In the other method hundreds of washed paramecia contained in 0.5 cc. of medium were placed in an experimental dish to which was added one washed *Woodruffia*. The latter type of culture will hereafter be referred to as a "completion culture." Completion cultures were prepared for tests at higher temperatures only (30–35° C.).

EXPERIMENTAL

It has already been demonstrated that a high percentage of unstable-cyst formation can be induced by increasing the concentration of salts in solution and that other factors such as moderately high temperatures, crowding, and starvation of food organisms contribute to a lesser degree in causing the production of such cysts. It would seem that the above conditions, which we have been able to produce in the laboratory with facility, would be considered as extreme when operating in nature, though possibly not infrequently encountered. Are unstable cysts formed under the influence of extreme conditions in general, or is there some underlying single cause?

I. HIGH TEMPERATURE

Earlier experiments on the effects of temperature on formation of unstable cysts have shown that there is a considerable increase in production of such cysts with increase in temperature when using completion cultures. In that study it was found that at 30° C. approximately 95 per cent of the cysts formed were unstable, and at 25° C. approximately 55 per cent. In an endeavor to repeat these results similar cultures were prepared using comparable food organisms. Precautions against evaporation were exercised. Table 1 shows the results. The formation of unstable cysts in the controls (25° C.) averages 62 per cent, and at 30°, 98 per cent. Completion cultures subjected to 35° C. show virtually 100 per cent formation of unstable cysts. At 35° C. the food organisms in the completion cultures had been consumed, and the *Woodruffia* were beginning the encystment process at the end of 1½ days; at 30° C. the time required was 2 days; and at 25° C., 3½ days. When the cysts in a given series were mature, generous samples were segregated, and the numbers of stable and unstable cysts counted; then the medium was removed, and distilled water added to test for activation.

TABLE 1

EFFECTS OF TEMPERATURE

(Completion cultures)

	Experimental						Control			
Series	1	2	3	4	5	6	1	2	3	4
No. of cultures	6	6	6	6	12	12	6	6	6	6
Temperature (° C.)	35	35	35	35	30	30	25	25	25	25
Percentage formation*	100	99+	99+	100	99	97	44	63	76	68
Percentage activation	100	100	100	100	100	100	85	87	90	81

* Percentage formation was determined by microscopical observation; percentage activation, by the excystment upon the addition of distilled water

Table 2 shows the results of experiments conducted to determine the effect of high temperature on uncrowded *Woodruffia* (30 per cubic centimeter) removed from their source of food and washed. The different series of experimentals and controls were run in parallel. In not a single case was unstable-cyst formation observed in the controls (25° C.). At 34° C. a few are formed, and the percentage rapidly increases as the temperature rises to 37° C., which is very close to the upper temperature limit of free-swimming *Woodruffia.* There is an apparent correlation between the percentage of unstable cysts and the percentage of activation, as though the presence of many such cysts somehow facilitated their excystment.

The use of completion cultures augments the formation of unstable cysts for a number of possible reasons. Crowding at the time of encystment, semistarvation of food organisms, increase in concentration of solutes in the medium by accumulation of metabolites, and the undisturbed condition of the *Woodruffia* at the onset of encystment no doubt have their influence. It has been our experience that agitation of *Woodruffia* by sucking in and out of a micropipette and thus delaying encystment for 3–5 hours tends to prevent the formation of unstable cysts; this was the primary reason for the use of washed *Woodruffia* in uncrowded cultures.

TABLE 2

EFFECTS OF HIGH TEMPERATURE

A. EXPERIMENTAL

Series	1	2	3	4	5	6	7	8	9	10
No. of cultures	6	6	6	6	6	6	6	6	6	6
Temperature (° C.)	34	34	34	35	35	36	36	36.5	36.5	37
Percentage formation	4.7	7.6	2 7	10.5	19	35	47	66	76	91
Percentage activation	3 2	7	.....	48	76	72	86	81	91	98

B. CONTROL

Series	1	2	3	4	5	6	7	8	9	10
No. of cultures	6	6	6	6	6	6	6	6	6	6
Temperature (° C.)	25	25	25	25	25	25	25	25	25	25
Percentage formation	0	0	0	0	0	0	0	0	0	0
Percentage activation	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....

II. LOW TEMPERATURE

Before the existence of unstable cysts was known, free-swimming *Woodruffia* were exposed to low temperatures in attempting to induce encystment by this means. It was found that organisms subjected to 10° C. formed cysts nearly twice the size of those formed at higher temperatures. It was our desire to find whether such cysts were unstable.

Experimental cultures of 30 *Woodruffia* each were placed in a 10° C. constant-temperature room. This low temperature slowed the encystment process markedly, and for this reason the cultures were left 14 days before removing for observation and testing. Table 3 reveals that all experimental dishes contained cysts of the unstable type; however, it was observed that certain differences from unstable cysts formed under other conditions existed. For example, the cyst wall is several times thicker, as measurements have shown (see Table 4). Both stable and unstable cysts formed at 10° C. show this same character of the wall. Another difference is the fact that unstable cysts formed at 10° C. require 3–4 hours for excystment, whereas such cysts produced under other conditions require but one-third the time. Excystment of 10° unstable cysts seldom occurs by substitution of distilled water for the encystment medium alone; but if the cysts in distilled water are transferred to a glass slide and a cover slip placed over them, good excystment usually takes place. No study of this problem has been made; however, pressure of the

TABLE 3

EFFECTS OF LOW TEMPERATURE (10° C.)

Series	Number of Trials	Average Percentage Formation	Average Percentage Death	Average Percentage Activation	Series	Number of Trials	Average Percentage Formation	Average Percentage Death	Average Percentage Activation
1	3	24	19 0	....	7	4	82	9 0	60
1*a**	1	0	0	....	8	4	83	15 0	10
2	2	75	10 5	....	9	4	58	16 0	0?
3	4	90	7.0	75	10	4	55	18 0	20
4	4	56	21 0	75	11	4	47	30 0	15
5	4	77	23 0	75	12	3	34	16 0	75
6	4	82	15 0	60	13	3	74	13 0	80

* Control at 25° C. Controls for the remainder of the series are not recorded, as the results were identical.

TABLE 4

MEASUREMENT OF CYSTS

ROOM-TEMPERATURE CYSTS		10° CYSTS	
Protoplast	Protoplast and Wall	Protoplast	Protoplast and Wall
50	77	95*	153
58	84	87	175
66	87	87	168
54	80	90*	190
80	102	77	160
58	77	87	153
67	80	109*	146
46	69	131*	175
58	77	75*	138
77	109	80	146
		102*	146
		109*	204
		131*	168
		116*	146
		116*	175
		109	182
		138*	197
		80	160
		95	131
		109*	138

* Unstable cysts.

cover slip may be a direct cause of excystment. It could not be induced by raising the temperature from 10° C. In all the 10° C. cultures some death occurred as recorded in the table. Controls at 25° C. showed neither unstable-cyst formation nor death.

III. HYDROGEN ION CONCENTRATION

Three sets of buffer mixtures were used, namely, acid potassium phthalate, pH 5.0–6.0; acid potassium phosphate, pH 6.0–8.0; boric acid–potassium chloride, pH 8.0–9.5 (Clark, 1925). The method for preparing the mixtures has been described elsewhere (Johnson and Evans, 1939). Determinations of H-ion concentrations were made with LaMotte's colorimetric standards.

Experimental animals were washed twice in the solution to be tested, and from the second wash 30 organisms were transferred with as little fluid as possible to the experimental dish containing 1 cc. of medium. Table 5 shows that in the vicinity of the acid-death point the formation of unstable cysts is greatest and that only within the narrow range of pH 5.6–6.0 were any such cysts formed. Alteration of the pH to neutrality was not effective in activating unstable cysts; addition of distilled water was necessary. Addition of organic medium (alfalfa extract) induced the excystment of stable cysts. Hydrogen ion concentrations less than a pH of 9.5 were not tested.

TABLE 5

EFFECTS OF H-ION CONCENTRATION ON FORMATION OF UNSTABLE CYSTS

pH	Trials	Average Percentage Formation	pH	Trials	Average Percentage Formation
5.0	4	Death in 1 hour	6.6	4	0
5.2	4	Death in 2 hours	7.0	4	0
5.4	4	Death in 24 hours	7.5	4	0
5.6	9	78	8.0	4	0
5.8	9	50	8.5	4	0
6.0	9	10	9.0	4	0
6.2	9	0.5*	9.5	4	0
6.4	4	0			

* Formation in only one dish.

IV. LOW OXYGEN TENSION

The fact that crowding, increasing the salt concentration, and increasing the temperature tend to facilitate the formation of unstable cysts and that a mere lowering of the temperature will induce them to excyst suggests that changes in oxygen tension may play a role both in the formation and in the activation of such cysts. If this relationship exists, only slight changes in oxygen tension would suffice in effecting the changes under consideration.

Experiments were devised to test this matter. An airtight system whereby *Woodruffia* could be maintained indefinitely in an atmosphere of nitrogen (99.7 per cent pure) or mixtures of air and nitrogen was set up. Two holes were bored through the lid of a Petri dish (diameter 10 cm.) on opposite sides, and an S-shaped glass tube sealed to each with De Khotinsky. The inlet to the Petri dish led from a liter Erlenmeyer flask filled with distilled water in which extended to the same depth two glass tubes (5 mm. inside bore). One of these admitted nitrogen (Linde high-test nitrogen), and the other, air under compression. By manipulation of valves the stream of bubbles of air and nitrogen could be regulated to give the desired proportions. The gas then passed to the chamber containing the experimental animals and thence to a 250-cc. Erlenmeyer flask where the escaping gas bubbled through water surfaced with mineral oil. With this setup it was possible to maintain the small cultures without any apparent decrease in volume of the medium over a period of 3 weeks.

At the beginning of an experiment a Columbia dish containing 40 washed experimental animals was placed in the Petri dish, and the two halves sealed together with hard paraffin wax. This assembly was put on the stage of a dissecting microscope. All joints of the system were also coated with the wax. Observations were made several times daily.

The first few trials were made by passing nitrogen unmixed with air through rapidly. The *Woodruffia* were dead within 3 hours in all these trials. In the following trials mixtures of air and nitrogen were used in varying proportions. When 1 bubble of air to 1 bubble of nitrogen was passed through the experimental chamber, the *Woodruffia* formed stable cysts in the normal time. The same was true when 1 bubble of air to 3 of nitrogen was tried. When 1 of air to 25 of nitrogen was tried, some of the organisms came to the surface of the encystment medium to encyst; but all formed stable cysts. All the organisms came to the surface to encyst when 1 bubble of air to 50 of nitrogen was tried; all cysts were stable. The same happened with 1 of air to 100 of nitrogen. In all the above experiments each *Woodruffia* divided once before encysting, and the encystment time was normal. When 1 bubble of air to 200 of nitrogen was passed through the experimental chamber, the encystment time was delayed approximately 1½ days, and the number of cysts totaled only 50; still all were stable. It has been found by Ephrussi, Chevillard, Mayer, and Plantefol (1929) that cell division of chick-embryo fibroblasts cultured *in vitro* was arrested at reduced oxygen tensions (7 mm. Hg.).

In the following trials the stream of nitrogen gas was slow but continuous (20–25 bubbles per minute) and the amount of air was limited to a few bubbles a day (no more than 30). When 30 bubbles of air were allowed to pass through, 1 week was required to complete encystment. The number of cysts amounted to 42, of which 20 were dead. Half of the living were unstable, and 30 per cent of these were activated by distilled water; excystment time was delayed as in the case of cysts formed at 10° C. In another trial using the same proportion of gases similar results were obtained. At the end of the experiment 39 cysts were present, 5 of which were dead; 54 per cent were unstable with 85 per cent activation. Four of the cysts had apparently activated without breaking through the cyst wall before completion of the experiment. All 4 had re-encysted to form the stable type. No activation could be induced by increasing the oxygen tension. All stable cysts formed under these conditions were viable when tested with the usual organic excystment medium.

When the amount of air was reduced to 20 bubbles per day, only 12 cysts remained alive, 10 of which were unstable. When the amount of air was reduced to 10 bubbles per day, all organisms were dead within 8 days.

It is obvious from the foregoing experiments that a much greater reduction in oxygen tension is required for the formation of unstable cysts than results from the crowding of organisms, the increasing of the salt content, or the raising of the temperature. Only a rough approximation of the oxygen tension is possible, but it can be said that unstable cysts were not formed until the percentage of oxygen in the air surrounding the culture lay at least below 0.1 of 1 per cent.

V. LOW OXYGEN TENSION WITH FOOD

In an earlier study (Johnson and Evans, 1939) it was found that of a wide variety of experimental conditions (quantity of food, pH, temperature, salt concentration, slow evaporation, crowding, metabolites, quality of food) only lack of food would induce the formation of protective cysts in *Woodruffia*. In these experiments the oxygen content had not been varied, and all the cultures were small in volume with a large surface area exposed. In the present work an attempt was made to control the oxygen tension by the method employed above. Each experimental dish at the beginning of an experiment contained 10 *Woodruffia* and hundreds of paramecia in 0.5 cc. of medium.

The first trial was made by passing 1 bubble of air to 10 bubbles of nitrogen through the chamber. During the first 3 days the fission rate amounted to about 1.3, whereas at that temperature a fission rate of at least 2.0 is to be expected. On the fifth day encystment of the *Woodruffia* began, even though food organisms were still numerous. Encystment was complete 2 days later; the number of food organisms had been reduced to one-tenth the number present at the beginning. There were about 1,500 cysts, 10 per cent of these being unstable as determined both by observation and by testing for excystment. The similarity of this culture to a completion culture probably accounts for the presence of unstable cysts. When 1 bubble of air to 20 of nitrogen was passed through the system, similar results were obtained; however, encystment began 1 day sooner. The fission rate amounted to 1.0. The number of food organisms remaining at the end of the cultural period was considerably more than in the first experiment. There were about 1,000 cysts, 3 per cent of which were unstable. In this case the similarity to a completion culture was lessened probably by the reduced oxygen tension.

When 1 bubble of air to 30 of nitrogen was used, the fission rate for the first 3 days fell to less then 0.45, and by the fifth day all had encysted to form 48 stable cysts. Any decrease in number of food organisms was not noticeable. This experiment was repeated with the formation of 65 stable cysts.

Then trials using 1 bubble of air to 60 of nitrogen were carried out. In the two tests tried, 19 and 22 cysts were formed respectively. Encystment commenced on the third day. In the first test 5 unstable and 4 dead cysts were found; in the second, all cysts were stable, but there was no death.

In the first trial using 1 bubble of air to 90 bubbles of nitrogen a leak appeared in the system on the third day, but during that time there was no division of the *Woodruffia* and no taking of food. The test was repeated. On the sixth day encystment began and on the eleventh day was completed. At the end of this period there were 12 cysts (indicating that only 2 of the 10 organisms divided before encystment), 5 of which were dead and 4 of the remaining were unstable. The three stable cysts were viable when tested with organic excystment medium. The duration of individual experiments varied between 7 and 20 days.

VI. AGING OF UNSTABLE CYSTS

A series of completion cultures was started at 30° C. to test the effects of age on viability. For the first 2 months, weekly tests were made on viability, the results of which have already been reported (Johnson and Evans, 1940). At the end of 2 months no change in viability had occurred. Thereafter, monthly tests were made, and at the end of 6 months the unstable cysts were still 100 per cent viable. It was noted, however, that with the passage of time the fluid vacuoles had increased both in number and in size. In all probability the end-result of this increased vacuolation will be the death of these encysted organisms.

DISCUSSION

According to the results obtained from the foregoing experiments, formation of unstable cysts seems to depend upon the occurrence of extreme conditions in general at the time of encystment. The existence of a single basic factor as the causal agent in the formation of such cysts appears to be unlikely. Since crowding, high temperature, and increased salt concentration facilitated unstable cyst formation, it was suggested that oxygen tension might be the single factor concerned; however, the oxygen tension required for the production of unstable cysts is so low that any such relationship does not appear likely. Another evidence against such a relationship is the fact that unstable cysts form at low temperatures. Among the various environmental conditions tested it was found that, as one approached the lethal point and as the percentage of deaths increased, the percentage of unstable cysts increased. Correlated with this was the increasingly prolonged encystment time, especially at low temperatures.

The finding here that decreased oxygen, as well as depletion of food, will induce encystment of *W. metabolica* even though ample food is present is in agreement with other workers. Rhumbler (1888) concludes that lack of air is a cause of encystment in *Colpoda*, though he describes no critical test. Adolph (1929) found that when the oxygen supply of *Colpoda* was suddenly removed, the organisms died; but when the removal was slower and not so complete some of the organisms formed permanent cysts, whereas none was formed in the controls. It was found by Taylor and Strickland (1938) that *Colpoda* are killed by lack of oxygen; however, it is their opinion that, if cyst formation can be induced by decreasing the oxygen tension, the direct factor is the inadequate supply of energy which is obtained from the food under these conditions. This situation may be true in the case of *Woodruffia.*

Amberson (1928) reports that the oxygen uptake of *Paramecium* is constant over a wide range (50–200 mm. Hg.) and that at 11 mm. Hg. the oxygen uptake is 80 per cent of that at atmospheric pressure. According to our experience, no effect of low oxygen tension was exhibited in *Woodruffia* until the partial pressure of the oxygen had been lowered to about 15 mm. Hg. As the pressure fell, the effect was augmented. Zoethout (1899) assumed that harmful oxidizable substances accumulate in the cell when deprived of oxygen. Such a situation might render the paramecia unfit as food, introducing, as a consequence, a condition comparable to absence or scarcity of food as far as the *Woodruffia* are concerned.

The lowest oxygen tensions seemed to have little or no apparent effect on the paramecia. Pütter (1905) found that paramecia remained living for as long as 10 days in some cases in the complete absence of oxygen. *Paramecium* has been found to inhabit the profundal bottom regions in lakes during stagnation periods of summer and winter (Welch, 1935). Here, according to analyses, no oxygen can be detected. Fauré-Frémiet, Léon, Mayer, and Plantefol (1929), by reducing the oxygen content by means of evacuation and also by means of reducing agents, found that *Paramecium* could survive up to

24 hours at 25° C. Kitching (1939) has reported that *Paramecium* will survive for 12 hours in anaerobic conditions produced by substituting especially purified hydrogen or nitrogen for atmospheric air. During this time decreasing activity and blistering was exhibited by the organisms. The organisms maintained a normal appearance at pressures as low as 1 mm. Hg.; death resulted at a partial pressure of oxygen of 0.2 mm. Hg.

The formation of permanent cysts in the presence of abundant food has been observed in one other situation. This phenomenon seemed to be associated with the use of old hay infusion as culture medium for *Woodruffia*. In these cultures the bottom of the dishes became matted with filamentous bacteria and fungi with other forms of microörganisms caught in the meshes. With such relatively enormous amounts of life in the small cultures it may be that the oxygen tension was lowered to a level insufficient to maintain the free-swimming *Woodruffia*. It was found that when the medium was filtered no such encystment occurred.

The true situation regarding the encystment of *Woodruffia* at low oxygen tensions when ample food is available cannot at present be certainly ascertained; however, three possibilities present themselves: (1) the effect on the *Woodruffia* may be direct, (2) the paramecia may not satisfy the energy requirements of the *Woodruffia*, and (3) the accumulation of harmful oxidizable substances in the food organisms may render them unfit as food. It is possible that all three factors may be operating simultaneously. However, the fact that the *Woodruffia* at the lowest oxygen tension did not remove enough of the food organisms to make any noticeable difference in their numbers favors the view either that there is a direct effect on the *Woodruffia* or that under these conditions the food organisms were so changed that they were no longer acceptable as food. In control cultures the same number of *Woodruffia* consume all the food organisms in the same period of time.

Cysts of different protozoa are known to remain viable for long periods of time. Stable cysts of *Woodruffia* (both wet and dry) were found to be viable after more than a year's time. Dawson and Hewitt (1931) report that dried cysts of *Colpoda* were viable after a period of 5 years and 4 months. Taylor in this laboratory has kept dried cysts of *Colpoda* for even longer periods, finding some loss of viability. Beers (1937) kept cysts of *Didinium* for 10 years with a gradual loss of viability. A good review of the literature is given in his paper.

Unstable cysts of *Woodruffia* cannot be dried without fatal results, so, for tests of aging, the cysts were maintained in the wet condition. Over the 6-month period no decrease in viability was observed. It was noted, however, that with the passage of time the fluid vacuoles increased considerably both in number and in volume. It seems probable that the source of energy necessary for the cytoplasmic movement which occurs in all unstable cysts is acquired by contributions from the substance of the body and that the diminution in the cytoplasmic volume is compensated by absorption of water. This condition would ultimately end in death of the cysts; and, according to an estimate of the cytoplasmic displacement, the life of the unstable cysts would be limited to but a few more months at most.

The viability of stable cysts decreases at a much slower rate. The metabolic activity transpiring in stable cysts appears to be slower than that in unstable cysts as adduced by the difference of movement between the two types. The situation in stable cysts seems to compare closely to that in cysts of *Colpoda* where the viability slowly falls.

SUMMARY

When free-swimming *Woodruffia* in absence of food were subjected to extremes of temperature (both high and low), high concentrations of H-ions, and low oxygen tensions, the formation of unstable cysts was induced. Under these extreme conditions a correlation between percentage of formation of such cysts and approach to the lethal point was noted. The cause of the production of unstable cysts could not be resolved to some single fundamental factor. The effect of low oxygen tension on feeding *Woodruffia* was tested and it was determined that as the oxygen tension fell the tendency to encyst increased, even though ample food was present. The direct cause for this phenomenon was not ascertained. The longevity of unstable cysts of *Woodruffia* was extended to 6 months with no decrease in viability.

LITERATURE CITED

ADOLPH, EDWARD F. 1929. The regulation of adult body size in the protozoan *Colpoda*. Jour. Exper. Zoöl., 53:269–311.

AMBERSON, W. R. 1928. The influence of oxygen tension upon the respiration of unicellular organisms. Biol. Bull., 55:79–91.

BEERS, C. DALE. 1937. The viability of ten-year-old *Didinium* cysts (Infusoria). Amer. Nat., 71:521–25.

CLARK, W. M. 1925. The determination of hydrogen ions. Baltimore: Williams & Wilkins.

DARBY, H. H. 1929. The effect of the hydrogen-ion concentration on the sequence of protozoan forms. Arch. f. Protistenk., 65:1–37.

DAWSON, J. A., and HEWITT, D. C. 1931. Longevity of encysted *Colpoda*. Amer. Nat., 65:181–86.

EPHRUSSI, B.; CHEVILLARD, L.; MAYER, A.; PLANTEFOL, L. 1929. Recherches sur le besoin d'oxygène libre. II. L'oxygène libre et les cultures de tissus. Ann. physiol. et physicochim. biol., 5:642–58.

FAURÉ-FRÉMIET, E.; LÉON, C.; MAYER, A ; and PLANTEFOL, L. 1929. Recherches sur le besoin d'oxygène libre. I. L'oxygène et les mouvements des paramécies. Ann. physiol. et physicochim. biol., 5:633–41.

GARNJOBST, LAURA. 1937. A comparative study of protoplasmic reorganization in two hypotrichous ciliates, *Stylonethes sterkii* and *Euplotes taylori*, with special reference to cystment. Arch. f. Protistenk., 89:317–81.

JOHNSON, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., 6:22–54.

JOHNSON, W. H., and EVANS, F. R. 1939. A study of encystment in the ciliate *Woodruffia metabolica*. Arch. f. Protistenk., 92:91–116.

———. 1940. Environmental factors affecting cystment in *Woodruffia metabolica*. Physiol. Zoöl., 13:102–21.

KIDDER, G. W., and STUART, C. A. 1939. Growth studies on ciliates. I. The role of bacteria in the growth and reproduction of *Colpoda*. Physiol. Zoöl., 12:329–40.

KITCHING, J. A. 1939. The effects of lack of oxygen and of low oxygen tensions on *Paramecium*. Biol. Bull., 77:339–53.

MOORE, E. L. 1924. Endomixis and encystment in *Spathidium*. Jour. Exper. Zoöl., 39:317–37.

PÜTTER, AUGUST. 1905. Die Atmung der Protozoen. Zeitschr. f. allg. Physiol., 5:566–612.

RHUMBLER, LUDWIG. 1888. Die verschiedenen Cystenbildungen und die Entwicklungsgeschichte der holotrichen Infusoriengattung *Colpoda*. Zeitschr. f. wissensch. Zool., 46:549–601.

TAYLOR, C. V., and STRICKLAND, A. G. R. 1938. Reactions of *Colpoda duodenaria* to environmental factors. I. Some factors influencing growth and encystment. Arch. f. Protistenk., 90:396–409.

WELCH, PAUL S. 1935. Limnology. New York: McGraw-Hill Co. P. 170.

ZOETHOUT, W. D. 1899. On some analogies between the physiological effects of high temperature, lack of oxygen, and certain poisons. Amer. Jour. Physiol., 2:220–42.

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FURTHER EXPERIMENTS ON ARTIFICIAL PARTHENOGENESIS IN STARFISH EGGS, WITH A REVIEW

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THE special physiological effect of fertilization or artificial activation in the resting egg cells of animals or plants is to initiate cell division, with the associated development, which may then proceed automatically to an advanced stage. It should be remembered, however, that effects of this general type are by no means confined to egg cells. The initiation of cell multiplication, growth, and differentiation in previously quiescent cells or tissues, as a result of some physical or chemical influence reaching them from their environment, is a widespread, if not universal, phenomenon in living organisms, illustrated more particularly in the various forms of response to developmental or morphogenetic stimuli. Every ontogeny, considered in its character as an integrated sequence of formative processes, depends on responses of this type. The inductive or organizing influence exercised by one region of the growing embryo on other regions includes such responses; and it is now known that the differentiation of the neural plate in the amphibian embryo may be initiated not only by the living cells of the dorsal lip of the blastopore but also by a variety of chemical and physical "stimuli" of a nonspecific kind. The morphogenetic stimulus may be of an "organizing" kind, or it may be a simple nonspecific initiatory or activating influence.[1] Artificial parthenogenesis, as a type of physiological process, falls in the latter category. The resting cell is aroused from a state of inactivity and proceeds to express its "latent" formative or developmental potentialities. Regenerative development and related phenomena, such as the production of supernumerary limbs in embryos as a result of trauma, may be regarded from a similar point of view (cf. Child, 1940, p. 38).

In higher animals definite chemical compounds, the hormones, acting singly or in combination, control the development, as well as the activity, of many special organs, especially those belonging to the reproductive system. The gonadotropic hormones of mammals and birds determine the special features of growth and differentiation, not only in the gonads themselves but also in accessory organs like the mammary gland and uterus, and the secondary sexual characters. In Amphibia the anterior pituitary hormone initiates cell division and differentiation in the early oögonia (Rugh, 1937), even though the development of these cells past the oöcyte stage requires fertilization or some other form of activation. The initiation of cell division is not the only phenomenon to be considered in activation processes of this type. Enlargement and differentiation of cells may occur independently of cell division; even in unfertilized egg cells differentiation may occur without cell division under certain activating treatments, as in the *Chaetopterus* egg (F. R. Lillie, 1902). The arrest of growth and development at a certain stage and their reinitiation by later physiological changes of condition are not infrequent in organisms, as illustrated, for example, in the dormant stages which occur during the metamorphosis of many animals. In the diapause of insects growth ceases temporarily, and respiration falls to a low level; both may afterward be renewed either under normal environmental conditions or as a result of certain kinds of artificial treatment (cf. Bodine, 1932; Bodine and Boell, 1934). The cells whose development is thus arrested have been called "blocked cells"; in a somewhat similar sense (as in certain characteristic features of their metabolism, such as insensitivity to cyanide) unfertilized egg cells (e.g., of Arbacia) correspond to blocked cells of a certain kind.

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[1] For a discussion of this difference cf. the recent book of Paul Weiss, *Principles of Development*, Part III (1939); see esp. pp. 366 ff.

Analogous phenomena occur in plants, where, also, artificial parthenogenesis is well known (for a recent study cf. Goddard, 1935). Lately the growth-promoting hormones of higher plants, the so-called "auxins," have been the subject of much investigation; these compounds may initiate cell division and growth in the cambium and elsewhere in the plant body, and they play an essential role in normal correlation. Like many animal hormones, they are compounds of known and comparatively simple constitution (for a review cf. the recent book of Went and Thimann, 1937).

Under certain conditions the initiation of cell division and proliferation in animals and plants may take on a pathological character. Growths may originate, under local chemical or other influence, which have no relation to the normal correlation or balance of parts and which are injurious or even destructive to the whole organism. But since auxins may "cause" galls and since compounds which are normal to animals, or near derivatives of these compounds, may produce tumors,[2] the pathological problem is not to be separated from the normal physiological problem. The parallels between tumor formation and the phenomena of artificial parthenogenesis have been discussed by many pathologists (see, especially, Leo Loeb, e.g., 1911, 1915, 1920).

From the foregoing brief discussion the broad biological interest attaching to the phenomena of activation in egg cells will be apparent. Fundamental problems of the physiology of growth and development are involved. In artificial parthenogenesis the physical and chemical conditions of activation can be studied in isolated cells of uniform constitution under exactly controlled conditions. The variety of parthenogenetic methods has proved to be surprisingly great, and no detailed description will be attempted in this article. A classified list was made by Harvey in 1910.[3] Since then there has been much additional research, but the recent interest has been not so much in the discovery of new methods or the application of old methods to new cases as in the physicochemical analysis of the conditions which underlie this sudden and remarkable transformation in the properties of the egg cell.

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[2] Cf. the large recent body of work on carcinogenic compounds. A recent review by S. E. Owen in *Quarterly Review of Biology*, 12 (1937), 340, discusses the biological relationships of these compounds.

[3] An extended summary of the earlier work is given in the article of E. Godlewski, Jr., "Physiologie der Zeugung," in Winterstein's Handbuch d. vergl. Physiologie, 3: Part II (1913), 805–67.

It is evident that the physiology of sperm fertilization is largely identical with that of artificial parthenogenesis, since activation is a feature of both; but in fertilization there is the additional factor of the fusion of two gametes with the accompanying genetic effects; also, the resulting activation is usually more uniform and complete than in artificial activation. Nevertheless, the latter may in favorable cases be followed by complete and apparently normal development, even in higher animals, such as the frog (Loeb and Bancroft, 1913; J. Loeb, 1920) and mammals (Pincus, 1939; Pincus and Shapiro, 1940). The initial deficiency in chromosomes may be partly responsible for the usually less favorable development in parthenogenesis, although in later stages this deficiency is apparently restored in many cases. (For chromosomes in artificial parthenogenesis of the frog see Parmenter, 1925, 1940; for a cytological study of the sea-urchin egg in early parthenogenetic development see E. B. Wilson, 1901).

The physiological analysis of the processes of artificial parthenogenesis began with the work of Jacques Loeb in 1899, although artificial activation of insect eggs had been studied earlier by Tichomiroff in 1886, and the initiation of cell division in unfertilized sea-urchin and annelid eggs by adding neutral salts to sea water (Loeb's first method) had already been accomplished by Mead and Morgan; Richard Hertwig also had obtained cell divisions in sea-urchin eggs by treatment with weak solutions of strychnine in sea water. A summary of the earlier work up to 1913 (with references to the work just cited) is given in Loeb's volume (1913). In his general discussion of factors (1913), based chiefly on his experience with the sea-urchin egg, Loeb assigned special importance to the additive or mutually supplementary effects of two distinct treatments separated in time. These were: (1) a surface effect produced by brief treatment with various, chiefly cytolytic, agents and associated with the separation of the "fertilization membrane," and (2) an internal metabolic effect involving oxygen consumption, lasting for a longer time ($\frac{1}{2}$–1 hour) and best produced by moderately hypertonic sea water (sea water with a 50–75 per cent increase in osmolar concentration produced by adding neutral salts or sugar). More recent studies have shown that the two separate treatments are not an essential feature of the activating procedure; even in sea-urchin eggs a single properly timed exposure to hypertonic sea water may give as complete an activation as a double treatment (Just, 1922*a*); and various types of single treatment (heat, acid, mechanical) have proved highly effective with other eggs (starfish, annelid, frog, mammal). Although we may concede that the internal factors immediately concerned in the initiation of development are multiple (as in any natural event), nevertheless it is an experimental fact that one simple external change of physical or chemical condition may, in many cases, so alter the conditions within the egg that it is transformed from the "resting" state, in which it sooner or later dies a "natural death" without further division or development, into an "active" state, in which it spontaneously begins and carries through more or less completely its normal cycle of development. Since the metabolic and other activity of the egg cell is thus profoundly modified by this temporary treatment, we conclude that all the factors required for development are already present or latent in the egg system but that their effective interaction is in some way prevented. Some slight change of condition is all that is needed to set in motion this process of interaction, which, once started, proceeds automatically. The critical initiatory event may be a shift of pH in the egg cortex, or on the physical side it may be an electrical or mechanical change; in some way it acts as a "releasing" factor and removes the block or hindrance to cell division and further development.

For the purposes of our present, mainly physicochemical, discussion we may describe the developmental process in general terms as consisting in a metabolic cycle of a specific kind, associated with the formation of definite structure and organization. The essential feature of the cycle is its coherence or integrated character; each step in the sequence follows automatically upon the preceding, and simultaneous processes are closely correlated or interlocked. The initiatory event in the sequence is that caused directly by the activating treatment. This event appears to be some chemical or metabolic reaction, possibly, as Loeb himself suggested, a splitting or hydrolytic reaction yielding some product or products which are utilized in later synthesis. Loeb's "corrective" process, induced by hypertonic sea water, was, in fact, regarded by him as consisting in the synthetic production of some substance necessary to further development (Loeb, 1913*a*, p. 119).

ACTIVATION IN THE STARFISH EGG

The conditions in the starfish egg indicate, more clearly than in most eggs, that activation is a progressive process, the primary stage of which consists in a metabolic reaction readily initiated by acid (or heat), which must proceed to a certain final stage if activation is to be complete. If the reaction stops short of this stage, activation is only partial; i.e., the eggs form membranes and cleave (usually slowly and irregularly) but soon cease development and disintegrate (R. S. Lillie, 1915). The most important fact about activation in this egg is its ready production by acids of all kinds. Apparently all nontoxic readily penetrating acids will activate this egg; and the rate of activation, within a considerable range of concentrations, is closely proportional to the concentration of acid (R. S. Lillie, 1914). With a given concentration of acid—e.g., 0.004 M butyric or other fatty acid dissolved in sea water—the proportion of eggs developing to a larval stage increases with duration of exposure up to a maximum and then decreases. A definite optimal duration of exposure is shown for each concentration of acid at a given temperature, and the curve relating duration of exposure to percentage of blastulae (or gastrulae) formed has a characteristic regular form, which is the same for heat activation as for acid activation. For this and other reasons it seems likely that heat activation[4] (by exposure to sea water at 30°–38° C.; R. S. Lillie, 1908, 1915) is, in reality, a special case of acid activation in which the acid is produced metabolically within the egg. Weak penetrating bases (ammonia) have no activating effect on the starfish egg. Since a partial activation resulting from brief exposure to acid or heat may be completed by a second properly timed exposure to either agent, as well as by hypertonic sea water (R. S. Lillie, 1915), the general inference seems justified that the accumulation of some reaction product to a certain level of concentration or quantity is the physiological requirement for complete activation. If the quantity of this product is too small (as with too brief exposure), the result is incomplete activation; if, on the contrary, it is produced in excess, activation is also defective, although for a different reason.

According to this interpretation, the primary or critical event in activation consists in a definite chemical reaction by which a substance necessary to development is produced within the egg. This hypothetical intermediary (comparable with a growth hormone or auxin) is called the "activating substance" (A). It is regarded as formed by interaction between two other substances, both of which are present in low or subthreshold concentration in the resting egg. One of these (S) is a synthetic product which may be increased in quantity by treating the egg in certain ways—e.g., by hypertonic sea water in the presence of oxygen; the other (B) is freed from pre-existing combination by a simple splitting process which is initiated or catalyzed by acid (i.e., by H-ions above a certain concentration); this reaction is independent of oxygen. The final activation reaction is symbolized by the equation $S + B = A$. The production of A may accordingly be promoted by increase in the available quantity (concentration or activity) of either S or B (or of both). This simple scheme is in correspondence with the experimental fact that the egg may be completely activated in two quite different ways, either (1) by a relatively prolonged exposure to oxygen-containing hypertonic sea water—which increases S—or (2) by a brief exposure to heat or acid under either aerobic or anaerobic conditions—which increases B. The egg may also be activated by a summation of the two treatments. In the sea-urchin egg complete activation by acid alone does not occur, and a supplementary action of hypertonic sea water[5] is required. In terms of the present hypothesis, this would mean that the resting sea-urchin egg, in comparison with the starfish egg, is deficient in the S-compound. In general, the dual nature of the process in the echinoderm egg is interpreted as indicating that two different types of metabolic reaction, respectively hydrolytic and synthetic, are concerned in activation; and it is assumed that the products of these reactions interact to produce a third substance, the activating substance (A), whose accumulation leads to activation (for a fuller discussion cf. R. S. Lillie, 1934).

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[4] The first successful experiments on heat activation in starfish eggs were those of Delage (1901).

Influence of hypertonic sea water on activation: General.—It is a fact of much general interest that a temporary moderate increase in the osmolar concentration of the surrounding medium should have an activating influence on the unfertilized eggs of so many animals. Treatment with hypertonic solutions, physiologically indifferent apart from their hypertonicity, appears to be the most generally effective parthenogenetic method. This treatment may be supplementary, as in Loeb's "improved method" (1913*a*, chap. viii) for the sea-urchin egg, or it may, by itself, be sufficient for complete activation. In the starfish egg, as in the sea-urchin egg, a partial activation by brief treatment with some membrane-forming agent, such as fatty acid or heat, may be completed by exposure to hypertonic sea water. A similar supplementary effect, however, may be produced by a second treatment with fatty acid or heat; and it is a special peculiarity of this egg that simply prolonging the exposure to either agent, in a single continuous exposure properly timed, will cause complete activation. This is not the case in the sea-urchin egg, where simple exposure to fatty acid produces only membrane separation, followed, perhaps, by a few cleavages; the same is true of other membrane-forming agents. On the other hand, a simple exposure to oxygen-containing hypertonic sea water of appropriate concentration may activate the sea-urchin egg uniformly and completely, as has been shown by Just (1922*a*) for *Arbacia;* this is true also for *Asterias*, although in my own experience with this egg a single treatment with hypertonic sea water has not usually proved so effective as one with heat or fatty acid (cf. R. S. Lillie, 1931, p. 302).

It is evident that in the sea-urchin egg the immediate physiological effect of hypertonic sea water is different from that of fatty acid or heat. Yet in the starfish egg any one of these three agents, acting alone, may cause complete activation; accordingly, they may be called the "efficient activating agents." There are also various forms of treatment whose activating effect seems never to be more than partial; shaking (Mathews, 1901), ultra-violet radiation (Lillie and Baskervill, 1922), photodynamic action (Lillie and Hinrichs, 1923), isotonic neutral salt solutions (R. S. Lillie, 1910, 1911; Dalcq, Pasteels, and Brachet, 1936), the electric current (Lillie and Cattell, 1925), and fat-solvent compounds (J. Loeb, 1913*a;* cf. p. 250) may all form membranes and induce cleavage in starfish eggs, and an occasional egg may develop to the blastula stage. But in the action of these agents there is always a deficiency that requires to be supplemented or "corrected" by an efficient agent. The purely physical action of hypertonic sea water is to abstract water osmotically from the egg, thus increasing the concentration of the egg constituents. In the absence of oxygen such a temporary loss of water seems to be physiologically indifferent; but when oxygen is present, certain secondary metabolic reactions follow which change the properties of the egg in the special manner characteristic of activation. In the sea-urchin egg some product or products of these metabolic reactions appear to be necessary for complete activation. Thus, in one of his experiments Loeb (1913*b*) found that a brief exposure of unfertilized *Strongylocentrotus* eggs to hypertonic sea water did not visibly alter the eggs or shorten their duration of life; yet for many hours after this treatment they remained in a state where they could be activated completely by a brief treatment with fatty acid. A natural inference is that some substance in which the egg was previously deficient has been formed; and this, in Loeb's view, is the basis of the "corrective" effect which he ascribed to the hypertonic treatment.

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[5] Loeb found that prolonged exposure to cold or cyanide could act, to some degree, as a substitute for hypertonic sea water.

In general, according to the mass-action rule, changes of concentration resulting from addition or abstraction of water may be expected to alter the conditions of chemical equilibrium within the egg; it is, therefore, not surprising that they should have metabolic and physiological effects. Since most protoplasmic syntheses, as of proteins from amino acids, are dehydrolytic, a partial dehydration would theoretically have the effect of shifting equilibrium in the direction of further synthesis. Such an increase in certain synthetic products necessary for development might conceivably be the basis for the widespread activating influence of hypertonic solutions.

Hypertonic sea water and activation in the starfish egg.—In the starfish egg a sufficiently prolonged exposure (½–2 hours at 20°) to oxygen-containing hypertonic sea water alone (beginning during the prematurational period) may cause complete activation in a large majority of eggs. The duration of the optimal exposures varies with concentration, decreasing with increase of concentration within a certain range. The starfish egg also responds, like the sea-urchin egg, to after-treatment with hypertonic sea water, following a brief initial treatment with fatty acid; but this effect varies with the length of the acid treatment and is favorable only with the briefer exposures to acid (R. S. Lillie, 1931).

Eggs exposed for moderate periods (10–30 minutes at 20°) to hypertonic sea water and then returned to normal sea water without further treatment show partial activation; they form membranes and, after a period of latency (longer than in the case of complete activation), may cleave a few times, but typically they break down early without further development. If, however, within 15 or 20 minutes after return to sea water they are given a second treatment, consisting of exposure for a brief period to a weak solution of a nontoxic penetrating acid in a balanced salt solution (e.g., 0.004 M fatty acid in sea water), they exhibit the typical progressive activation with a maximal production of blastulae at a certain well-defined duration of exposure; this optimal exposure, however, is briefer than that shown by eggs with no previous hypertonic treatment. The eggs may be described as having been "sensitized" by the hypertonic sea water to the activating influence of the acid; i.e., the activation reaction induced by the acid proceeds in the usual manner but at an increased rate. The degree of this acceleration varies (at constant temperature) with the concentration of the hypertonic sea water and the duration of the exposure. With hypertonic sea water of constant composition (100 vols. sea water *plus* 20 vols. 2.5 M NaCl) the curve relating degree of sensitization to duration of previous exposure shows a good correspondence with that of a typical bimolecular reaction (R. S. Lillie, 1934). This is consistent with the hypothesis that the sensitization (equivalent to partial activation) results from the formation of an activating substance *A* by the union of two precursors *B* and *S*, of which *S* is increased in quantity by the hypertonic treatment and *B* by the acid treatment.

TABLE 1

June 29, 1934. Eggs from several starfish were exposed during the prematurational period to hypertonic sea water for 20 minutes at 20°–21° C. and returned to normal sea water. After 18 minutes in sea water, they were transferred to a 0.004 M solution of butyric acid in sea water; from this solution portions were returned at 1-minute intervals to normal sea water. Next day the proportion of eggs developing to a blastula stage with each duration of exposure was determined.

Concentration of Hypertonic Sea Water (Vols. 2.5 M Na-Ca Added to 100 Vols. Sea Water)	Duration of Optimal Exposure and Approximate Percentages of Eggs Developing to Blastula Stage	
A. 0 (control)	6–7 min.	(85–90 per cent)
B. 10	5–6	(70–80)
C. 20	*ca.* 4	(50–60)
D. 30	*ca.* 4	(25–35)

Influence of concentration.—In experiments carried out chiefly during the summers of 1934 and 1935, I have studied in some detail the relation between the concentration of the hypertonic sea water and the degree of sensitizing action. The sea water was made hypertonic by the addition of a strong partly balanced salt solution consisting of 95 volumes of 2.5 M NaCl *plus* 5 volumes of 2.5 M $CaCl_2$ (called, in the following, "2.5 M Na-Ca").

There is a lower limit of effective hypertonicity corresponding approximately to the mixture of 10 volumes of 2.5 M Na-Ca *plus* 100 volumes of sea water. The mixture of 5 volumes of 2.5 M Na-Ca *plus* 100 volumes of sea water has no apparent effect. In experiments with additions ranging from 10 to 30 volumes of 2.5 M Na-Ca to 100 volumes of sea water the effect is distinct, and its rate (as shown by the sensitization produced by a constant exposure) increases with concentration. Hypertonicity beyond this range is quickly injurious to the eggs.

A typical protocol (June 29, 1934), as given in Table 1, shows the general manner in which the rate of activation increases with increase in the concentration of hypertonic sea water. In this experiment the control eggs (*A*), treated with 0.004 M butyric acid alone, showed optimal development with 6–7 minutes' exposure. The effect of the previous stay in hypertonic sea water is seen in a shortening of the optimal exposure; this effect increases with concentration.

Table 2 summarizes the results of a considerable number (33 in all) of similar experiments carried out during these two summers. The arithmetical means of the optimal activation times of experiments conducted under similar conditions are given. Usually the optimal exposure to 0.004 M butyric acid (in sea water) at 20°–21°, without any previous treatment with hypertonic sea water, lies between 6 and 8 minutes, varying somewhat with the animals and with the season of the year. Often in a single experiment the time curve of activation may have a flattened top; i.e., two (or even three) exposures separated by intervals of 1 minute (e.g., 6 and 7 minutes) may appear equally favorable, as in Experiment *A* above. In computing the averages, the optimal exposure in any such case is taken as the mean of the two (or more) equally favorable exposures (i.e., 6.5 minutes in the example cited). In all experiments the exposures to hypertonic sea water lasted 20–21 minutes, and the temperature was 20°–22°.

TABLE 2

In all experiments the eggs were exposed during the prematurational period to the hypertonic sea water at room temperature (20°–22° C.) for 20–21 minutes; then, after 15–20 minutes in sea water, they were transferred to 0.004 M butyric acid in sea water at the same temperature. Portions were then returned to normal sea water at intervals of 1 minute. (The number of separate experiments with each concentration of hypertonic sea water is indicated in brackets in the first column. Probable errors by Peter's approximation formula.)

Hypertonic Sea Water (Vols. 2.5 M Na-Ca Added to 100 Vols. Sea Water)	Average Optimal Exposure for Control Eggs Treated with Acid Alone (Minutes)	Average Optimal Exposure for Eggs Treated also with Hypertonic Sea Water	Percentage of Decrease in Optima with Hypertonic Treatment (Nearest Whole Number)
5 (5)	6.0±0.51	5.9±0.47	0
10 (7)	6.5±0.40	5.7±0.37	12
15 (9)	7.5±0.46	6.1±0.39	18
20 (6)	8.2±0.44	5.9±0.34	28
25 (3)	9.2±0.14	6.2±1.0	33
30 (2)	6.5±0	4.2±0.21	35

As already mentioned, Loeb's view of the dual nature of the activation process was based on the observed difference in the mode of action of the two treatments, both of which he found necessary for complete activation in the sea-urchin egg. One of these acts superficially, is rapid, and is independent of respiration; the other (called "corrective") occurs while the egg is in the hypertonic sea water; it is more prolonged and requires the presence of oxygen. The fact that hypertonic sea water, acting alone, may activate sea-urchin eggs completely has been considered inconsistent with Loeb's interpretation (Just, 1922*a*), but such a criticism seems hardly justified. In the starfish egg, also, the mutually supplementary action of the two treatments is shown; yet either, acting alone, may cause complete activation. There is, however, no inconsistency if activation is regarded as dependent in the union of two substances, *S* and *B*, both of which are present in low or subthreshold concentration at the site of activation and in equilibrium with the activating substance, *A*, formed by their combination. According to this view, the accumulation of A to the critical level required for complete activation is the essential feature of the process. Experiment shows that activation is a progressive process which may be stopped at any stage short of completion and later be renewed. The incompletely activated egg behaves as if it were deficient in some material necessary for development. By the mass-action rule a sufficient increase in either S (formed by hypertonic sea water) or B (freed by some splitting process promoted by acid) would lead to a sufficient production of A, and so to activation. The sequence of metabolic reactions started by activation is necessarily complex, since their consequence is development; but it seems clear that the initial or releasing reaction of this sequence may require only a slight change of equilibrium. This primary or critical change is to be regarded as definitely localized in the cytoplasm of the egg, in a region which apparently is to be identified with the most superficial layer or cortical region (Chambers, 1921*a*, 1921*b*). Mrs. Harvey's recent experiments on parthenogenetic merogony (E. B. Harvey, 1936, 1940) furnish additional and crucial evidence that activation is a cytoplasmic reaction independent of immediate nuclear influence.

Rate of activation reaction as directly influenced by hypertonic sea water.—The purely physical effect produced by hypertonic sea water is withdrawal of water. It should be noted that this effect must occur most rapidly in the superficial region of the egg, corresponding to the so-called "cortical region." Although 10–15 minutes elapse before the whole egg reaches osmotic equilibrium with its surroundings (cf. Stewart and Jacobs, 1932), this entire period corresponds to the time required for an osmolar concentration equal to that of the hypertonic sea water to be attained within the whole volume of the egg. During the time occupied by the osmotic movement of water, there is a gradient of concentration from the external to the internal regions of the egg, with the highest concentration at the surface. Accordingly, when the egg is placed in the hypertonic sea water, a thin layer of protoplasm immediately inside the plasma membrane will almost instantly attain osmotic equilibrium with the surroundings, and a flow of water from within outward will continue as long as the internal regions are more dilute than the external. Transfer of water then ceases, and the whole system is in equilibrium.

The activating effect of such a temporary increase of concentration is dependent on oxidative metabolism, as we have seen, and is relatively gradual. Eggs remaining in this partly dehydrated condition for 10–20 minutes show an increased rate of activation when, after a brief interval in sea water (during which they regain their normal water content), they are placed in acid-containing sea water (sensitization effect; Table 1). If, however, they are placed in this solution directly, i.e., without the interval in sea water—or while the egg cortex is still in a water-depleted state—the acceleration is much less, or activation may even be retarded instead of accelerated. Apparently, the water content of the egg at the actual time of activation influences the rate of the reaction directly. Conversely, it is found that the rate of activation is increased by addition of water to the egg, as when activation occurs in dilute sea water or after brief exposure to dilute sea water (see below). It may seem surprising that, while an exposure of 20 or 30 minutes to hypertonic sea water brings the eggs into a state in which, after regaining their normal water content, they show an accelerated response to acid or heat, the activation reaction itself is retarded if it occurs *while* the eggs are still in the partly dehydrated condition. Apparently this means that a decrease in the water content of the protoplasmic system has in itself a delaying effect on the progress of the activation reaction. Since dilution has the opposite effect, the inference seems natural that the rate at which the precursors (symbolized by *B* and *S* of our scheme) unite to form the activating compound (*A*) is determined (in part) by the water content, i.e., the structural density, of the protoplasm at the site of the reaction, the cortical region. Apparently, increased density slows diffusion and hence rate of interaction, while decreased density has the reverse effect.

To illustrate this directly retarding effect of partial dehydration, an experiment described in a former paper may be cited (R. S. Lillie, 1934, p. 372). Two portions of the same lot of eggs transferred simultaneously from normal sea water to two 0.004 M solutions of butyric acid—one (*A*) in normal sea water, the other (*B*) in hypertonic sea water (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca)—showed optimal exposures (with 80–90 per cent of blastulae) at 8 minutes in *A* and at 14 minutes (or longer) in *B*. This result is typical and has been confirmed many times under a variety of conditions.

TABLE 3

July 17, 1935. Mixed eggs from several starfish were used; 95 per cent or more maturated; sperm-fertilized controls are normal. All treatments were during prematurational period.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in sea water	Optimal exposure at 5–6 min. (*ca.* 50 per cent blastulae)
B. Eggs exposed to hypertonic sea water (100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca) for 25 min.; then to normal sea water for 17 min.; then to 0.004 M butyric acid in sea water	Good activation with optimum at 4–5 min. (70–80 per cent blastulae)
C. Same treatment with hypertonic sea water, but eggs were transferred directly to 0.004 M butyric acid in sea water	Also good activation with optimum at 6 min. (70–80 per cent blastulae)

In experiments of the type summarized in Table 2, the interval in normal sea water allows the eggs time to regain their normal water content; activation is then found to be definitely more rapid than in untreated eggs. The experiment described in Table 3, in which a less concentrated hypertonic sea water (100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca) was used, shows the effect of omitting the interval in normal sea water. In this experiment the eggs treated first with hypertonic sea water and then returned to normal sea water for an interval (*B*) show the usual increased rate of activation; while those transferred directly to the acid-containing sea water (*C*) show no acceleration but rather a slight retardation. Experiments with more concentrated hypertonic sea water (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca) gave the same result; but in such cases the effect of direct transfer to the acid-containing sea water is injurious, probably because of the greater osmotic change, and few eggs develop to the blastula stage.

In experiments of this type (*C*) the activation reaction begins while the eggs are still in a partly dehydrated state, and the retarding effect of this condition shows itself in a partial counteraction of the acceleration which otherwise would be expected from the action of the hypertonic sea water. But since the osmotic entrance of water into the cortical layer begins immediately after the eggs are transferred to the acid-containing sea water, the degree of counteraction is relatively slight. When this osmotic change is avoided, as by bringing the eggs from hypertonic sea water directly into a solution of acid *made up in the same medium*, the retardation is much greater, and it increases rapidly with increase in hypertonicity. Many experiments of this kind have been carried out during several seasons; and the results, while showing considerable quantitative variation, have been entirely uniform as regards the direction of the effect.

The experiment described in Table 4 is typical and shows the increase of retardation with increase in hypertonicity. In this experiment the eggs were exposed to neutral hypertonic sea water for ½ hour before being exposed to acid. In other experiments, however, the duration of the previous hypertonic treatment was found, somewhat unexpectedly, to make little or no difference in the rate of activation in the acid-containing solution. An experiment comparing the influence of brief and longer exposures to hypertonic sea water is given in Table 5. As already described, retardation is shown if eggs are transferred directly from normal sea water to acid-containing hypertonic sea water, although typically the retardation observed under such conditions is less than with a previous hypertonic treatment. The relative absence of a time influence is understood if the activation reaction is considered as having its site in the cortical layer of the egg, where the osmotic loss of water occurs most rapidly.

TABLE 4

August 5, 1936. Good lot of eggs; great majority show normal maturation and develop normally on fertilization.

Treatment	Result
A. (Control.) 0.004 M butyric acid in sea water. Temperature 22° C.	Optimum at 5 min. *Ca.* 90 per cent of mature eggs form blastulae
B. Eggs exposed to hypertonic sea water (100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca) for 32 min., then to 0.004 M butyric acid in the same solution	Retardation distinct but moderate; optimum at 6 min., with *ca.* 50 per cent blastulae
C. Treatment as in *B*, but the hypertonic sea water is more concentrated (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca)	Marked retardation; optimum at 9 min. with 80–90 per cent blastulae; 70–80 per cent at 11 min. (the longest exposure)

The average retardations (expressed in terms of increase in optimal exposure) observed in a considerable number of experiments during 1936 and 1937, with previous exposures of 16–35 minutes to hypertonic sea water of two concentrations, are shown in Table 6. The concentration of butyric acid was 0.004 M in all cases; and the temperature of the acid solutions, 20°–22°. The number of experiments with each concentration is indicated in the first column, in brackets.

From these experiments it is clear that the activation reaction induced by fatty acid proceeds at a decreased velocity in eggs which are already in a state of partial dehydration under the influence of hypertonic sea water. In the terms of our hypothesis the velocity of the reaction $S + B = A$ is decreased. Experiments on the heat activation of starfish eggs also show a decreased rate of reaction when the eggs are exposed to activating temperatures while immersed in hypertonic sea water. Thirteen experiments of this kind were performed during the summers of 1938 and 1939; the eggs were exposed (A) to normal and (B) to hypertonic sea water at 32° simultaneously and under otherwise similar conditions, without previous hypertonic treatment. The sudden transfer from normal sea water at 20° to hypertonic sea water at 32° appears to be injurious to the eggs, and blastulae were usually fewer than in the control (normal sea water at 32°). Lower concentrations of hypertonic sea water were used, with additions of 2.5 M Na-Ca ranging between 1.5 and 10 volumes to 100 volumes of sea water. Under these conditions a large proportion of eggs may form blastulae; and retardation, while moderate, is distinct, especially in the mixtures with additions of 5–10 volumes of 2.5 M Na-Ca. Membrane separation is especially subject to hindrance under these conditions; typically, about 3 minutes' exposure to normal sea water at 32° is required to form membranes in a ma-

TABLE 5

July 23, 1937. Most eggs show normal maturation; sperm-fertilized controls are normal.

Treatment	Result
A. (Control.) 0.004 M butyric acid in sea water. Temperature 21°–22° C.	Optimum at 8 min.; *ca.* 50 per cent of mature eggs form blastulae
B. Brief treatment of *ca.* 1 min. with hypertonic sea water (100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca); thence to 0.004 M butyric acid in the same medium	Retarded activation with optimum at 11 min. (*ca.* 50 per cent blastulae)
C. 20 minutes in the same hypertonic sea water as in *B*; otherwise similar	Retarded activation with optimum at 10–11 min. (20–30 per cent blastulae)

TABLE 6*

Hypertonic Sea Water	Average Optimum for Control (Minutes)	Average Optimum with Hypertonic Treatment	Percentage of Increase in Duration of Optimum over Control
100 vols. sea water *plus* 10 vols. 2.5 M Na-Ca (4)....	5.6±0 55	6.9±0.55	23
100 vols. sea water *plus* 20 vols. 2.5 M Na-Ca (17)....	7 5±0.27	11.3±0.37	50

* In 1 experiment with less concentrated hypertonic sea water (100 vols. sea water *plus* 5 vols. 2.5 M Na Ca) no retardation was shown. In 2 experiments with an intermediate concentration (100+15) the optima for the controls were 9 and 5 minutes, respectively, for the treated eggs 13 and 7 minutes.

jority of eggs (R. S. Lillie, 1915; cf. p. 274); but in the hypertonic mixtures with 5 volumes of 2.5 M Na-Ca the time was prolonged to 5 minutes or more, and even the mixture with 1.25 volumes had an appreciable effect.

Influence of dilute sea water on activation.—In contrast to hypertonic sea water, a temporary exposure of starfish eggs to moderately dilute (hypotonic) sea water has no definite effect in altering the rate of subsequent activation by fatty acid. There is no evidence of retardation, i.e., of an effect opposite in character to the typical acceleration or sensitization following temporary hypertonic treatment. No immediate activating effect is seen, not even membrane formation. Apparently the intake of water is perfectly reversible; and the eggs remain unaffected in their ability to respond later to artificial activation or sperm fertilization. This absence of effect was noted in my 1934 paper and has been repeatedly confirmed in more recent experiments.

It should be noted, however, that in the eggs of some other species of animals hypotonic solutions have proved to be remarkably effective parthenogenetic agents. This is notably the case in the egg of the annelid *Urechis*, as recently shown by Tyler (1931*a*, 1931*b*, 1932); and recently Pincus and Shapiro (1940) have found dilute serum or hypotonic balanced salt solution highly favorable with mammalian eggs.

In the experiments with temporary hypotonic treatment dilutions of sea water ranging between 90 volumes per cent (90 vols. sea water *plus* 10 vols. distilled water) and 60 volumes per cent were used, with exposures of 20–35 minutes, followed after an interval of 10 or 15 minutes in sea water by exposure to 0.004 M butyric acid in sea water. Since the results were uniformly negative, they may be summarized briefly. With the higher dilutions (60–70 vols. per cent), a slight acceleration (shortening of the optimal exposures to acid) was observed in several experiments, but the lower dilutions appeared quite indifferent. Some slight activating or sensitizing effect seems indicated in the higher dilutions, although no fertilization membranes were formed; but, in general, the absence of any decided influence in either direction is in striking contrast with the definite action of the hypertonic medium.

On the other hand, if starfish eggs are activated by fatty acid or heat *during* the immersion in dilute sea water, the rate of activation is definitely accelerated; this effect is appreciable in low dilutions and increases rapidly as the dilution is increased. In typical experiments of this kind the eggs are first exposed to dilute sea water and then transferred directly to a solution of butyric acid made up in the same medium. If, after the preliminary exposure to dilute sea water, the eggs are transferred to a solution of the butyric acid in normal sea water, they also show an increased rate of activation, but the effect is relatively slight (as in the analogous experiments with concentrated sea water) and is distinct only with the higher dilutions. This is shown in the experiment described in Table 7. Activation is definitely but moderately accelerated after the 70 volumes per cent dilution, and still more so after the 60 volumes per cent, but the higher dilution is injurious.

If, however, the eggs, after the exposure to dilute sea water, are transferred directly to 0.004 M butyric acid in the same dilute sea water, acceleration is much greater. The experiment described in Table 8 will illustrate. In this experiment activation occurs at twice the rate in the dilute as in the normal sea water, with the same lot of eggs and the same previous exposure to dilute sea water.

As in the case of hypertonic sea water, the duration of the previous stay in the dilute sea water has little influence on the rate of activation in the acid-containing dilute sea water. This was shown in experiments in which the eggs were previously exposed to dilute sea water for periods varying from 1 minute (about the least time possible with the necessary manipulation) to 35 minutes. A typical protocol is given in Table 9. The rate of activation is greatly increased with both the brief and the longer exposures but is only slightly greater in the latter case. Eggs transferred directly from normal sea water to the 0.004 M solution of butyric acid in 80 volumes per cent sea water also show an increased rate of activation, but this treatment is injurious to the eggs, and relatively few reach the blastula stage.

TABLE 7

July 13, 1931. Most eggs (75–85 per cent) show normal maturation and develop normally on fertilization.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in sea water. Temperature 22° C.	Good activation with optimum at 6 min. (65–75 per cent blastulae)
B. Eggs in 80 vols. per cent sea water for 28 min.; thence to 0.004 M acid in sea water as in *A*	Very like control; optimum at 6 min. (70–80 per cent blastulae)
C. Eggs in 70 vols. per cent sea water 28 min.; thence to acid sea water as above	Moderate acceleration; optimum at *ca.* 5 min. (55–65 per cent blastulae; at 6 min. *ca.* 50 per cent)
D. Eggs in 60 vols. per cent sea water 28 min.; thence to acid sea water as above	Treatment is injurious; relatively few eggs form blastulae; optimum at 4 min. (*ca.* 20 per cent blastulae)

TABLE 8

July 30, 1936. Eggs show 90 per cent maturation and develop normally after fertilization.

Treatment	Result
A. (Control.) Eggs exposed to 0.004 M butyric acid in sea water (22.5° C.)	Typical activation with optimum at 6–7 min. (65–75 per cent blastulae)
B. Eggs in 75 vols. per cent sea water for 32 min.; then to 0.004 M butyric acid in normal sea water	Typical activation with moderate acceleration; optimum at 4–5 min. (80–90 per cent blastulae)
C. Similar to *B*, but with 0.004 M butyric acid in 75 vols. per cent sea water	Marked acceleration with optimum at 2–3 min. (60–70 per cent blastulae; eggs are overexposed at 4 min. and form no blastulae)

During the summers of 1936 and 1937 a considerable number of experiments (29 in all) were made in which eggs were exposed for longer periods, of 15–36 minutes, to dilute sea water and then activated by direct transfer to 0.004 M butyric acid in the same medium. Omitting four of these (in which the proportion of good eggs was small), the average optimal durations of exposure in four dilutions were as given in Table 10, third column.

TABLE 9

July 28, 1937. Mixed eggs from 2 starfish; 80–90 per cent maturation. Normal development on fertilization.

	Treatment	Result
A.	(Control.) Eggs exposed to 0.004 M butyric acid in normal sea water (21°–22° C.)	Typical activation with optimum at 6–7 min. (70–80 per cent blastulae)
B.	Eggs in 80 vols. per cent sea water for 1 min., 5 sec.; then to 0.004 M butyric acid in 80 vols. per cent sea water	Optimum at 3 min. (*ca.* 50 per cent blastulae)
C.	Eggs in 80 vols. per cent sea water for 20 min.; then to 0.004 M butyric acid in 80 vols. per cent sea water	Optimum at 2–3 min. (2 min.: 60–70 per cent; 3 min.: 70–80 per cent blastulae)

TABLE 10

The first column gives the dilutions used, with the corresponding number of experiments in brackets;* the second column, the average optima for the controls (0.004 M butyric acid in normal sea water); the third column, the average optima in dilute sea water; the fourth column, the average decrease in these optima, expressed as percentages of the optima in the controls.

Dilution (Vols. Per Cent Sea Water)	Optima in Controls (Minutes)	Optima in Dilute Sea Water	Percentage of Decrease
90 (5)	6.4±0.66	4.6±0.39	28
85 (5)	6.5±0.67	4.1±0.50	37
80 (7)	6.6±0.27	3.3±0.26	50
75 (8)	8.0±0.44	3.5±0.30	56

* Two similar experiments with 70 vols. per cent sea water gave optima in the controls of 8 minutes and 6 minutes, and in the dilute sea water of 3 minutes and 2 minutes, an average decrease of 64 per cent.

The preliminary exposure to dilute sea water, even if brief, is an important part of the treatment, since a direct transfer from normal sea water to the acid-containing dilute sea water is highly injurious. In a typical experiment (July 23, 1936), eggs which yielded 80–90 per cent of blastulae with an optimal exposure of 10 minutes to 0.004 M butyric acid in sea water gave only 2 or 3 per cent, with an optimum at 4 minutes, when transferred directly to 75 volumes per cent sea water containing the same concentration of acid. Eggs of the same lot previously exposed for ½ hour to dilute sea water showed also an optimum at 4 minutes, but the great majority (65–75 per cent) formed blastulae. It would seem that when the action of the acid occurs simultaneously with the sudden osmotic entrance of water into the surface layer of the egg an injurious effect results which is absent when the surface layer is already diluted before the entrance of the acid. As in the analogous case of hypertonic sea water, the rapidity with which the dilution effect appears is evidence that the activation reaction occurs in the cortical layer of the egg.

Heat activation is also accelerated when the eggs are warmed to 32° in dilute sea water, either with or without a previous exposure to the dilute sea water; but the degree of acceleration has proved to be somewhat less than with acid activation. A typical experiment with direct transfer from normal sea water to warm sea water, both normal and dilute, is given in Table 11.

In a typical experiment with eggs previously exposed to a somewhat highly dilute sea water (65 vols. per cent) for 23 minutes and then transferred to the same medium at 32°, a well-defined optimum was shown at 3 minutes (*ca.* 50 per cent blastulae); while in the control (normal sea water at 32°) the optimum was 5 minutes. In the warm normal sea water the time required to form membranes in a majority of eggs was 3 minutes; this was shortened to 2 minutes (or less) in the dilute sea water. The same facilitation of membrane separation was found uniformly in all the experiments in which eggs were warmed in the dilute medium. In general, all the typical effects which result from treatment with activating agents (acid as well as heat) appear more rapidly in the dilute medium. This is true, for example, of the injury following exposures longer than the optimal. Thus, in the experiment just cited the eggs warmed to 32° in the dilute sea water for 5 minutes showed all the effects of overexposure (early disintegration with no blastulae formed), while the same exposure was optimal for the eggs warmed in normal sea water. It is as if a single metabolic reaction, on which the other physiological effects of activation depend, proceeds at an increased rate in the diluted protoplasmic layer.

TABLE 11

June 22, 1938. Eggs from 2 starfish; more than 90 per cent maturate. Fertilized control normal.

Treatment	Result
A. (Control.) Normal sea water at 32° C.	Optimum at 5–6 min. with 80–90 per cent blastulae
B. 90 vols. per cent sea water at 32°	Optimum at 4–5 min. with 70–80 per cent blastulae
C. 85 vols. per cent sea water at 32°	Optimum at 4 min. with 70–80 per cent blastulae

It should again be noted that it is only while the egg protoplasm is in the diluted state that this facilitation is observed. Experiments in which eggs are returned from the dilute to normal sea water before treating with acid show the complete reversibility of the effects of dilution. It appears, therefore, that the velocity of the activation reaction is determined (in part) by the water content, i.e., the structural density, of the egg protoplasm and varies reversibly with this. The opposite effect, retardation, is seen in the experiments where the eggs are activated in hypertonic sea water. Hypertonic sea water, however, has an additional special activating effect in the presence of oxygen; this action, as already described, proceeds gradually and is referred above to an oxygen-dependent metabolic reaction by which a special substance, S, is synthesized. But in the absence of oxygen (or presence of cyanide) the effect of hypertonic sea water appears to be a purely physical and osmotic one and to be completely reversible in the same sense as the effect of dilute sea water. The fact that simply changing the water content of the egg alters the velocity, but not the essential character, of the progressive reaction by which the egg is brought from the resting into the active state is evidence that the structural density of the protoplasmic gel in the egg cortex controls the rate of union of the reactants (the hypothetical B and S of our scheme) to form the activating substance (A). It is known that the rate of diffusion of large molecules in gels—a factor which limits their rate of chemical interaction under such conditions—varies with the density of the gel in the same general manner (cf., e.g., Kraemer, 1931, p. 1711; Freundlich, 1923, p. 727).

Other conditions influencing the velocity of the activation reaction in starfish eggs.—Temperature is a general physical condition which appears to influence the rate of activation in two different ways. At a lower range of temperatures, up to 20°, activation in butyric acid solutions in sea water shows the usual chemical type of temperature coefficient ($Q_{10} = ca.\ 3$); but at higher temperatures the Q_{10} values increase rapidly (R. S. Lillie, 1917), indicating apparently the superposition of an incipient heat-activation effect. This effect, in its turn, is probably to be referred to the production of additional acid within the egg. The activating effect of cold on the starfish egg (Greeley, 1902) is a special phenomenon peculiar to this egg, referable possibly to some structural (e.g., colloidal) change in the egg cortex, analogous to that caused by mechanical treatment.[6] This structural change initiates the activation reaction, presumably through the production of acid. Activation by butyric acid at low temperatures (2°–6°) is more rapid than would be expected from a consideration of temperature conditions alone (R. S. Lillie, 1917), indicating a summation effect analogous to that observed at high temperatures.

The velocity and character of the activation reaction in acid sea water are not immediately changed by lack of oxygen or presence of cyanide; but, if the eggs are kept under anaerobic conditions for some time before they are activated, an acceleration is shown, apparently as a result of increased acid production within the egg (R. S. Lillie, 1931). Activation by heat or acid may be influenced by other types of chemical condition; thus, certain compounds may increase the rate of activation by their mere presence, independently of any direct activating influence of their own. An interesting example, observed some years ago, is the acceleration in weak solutions of ethyl alcohol (R. S. Lillie, 1914, 1917). In sea water containing 2–3 volumes per cent of alcohol the velocity of both heat and acid activation is approximately doubled. Activation by cold (2°–4°) is also promoted by alcohol in a striking way. Since alcohol alone causes no appreciable activation in these concentrations, some physical effect (such as displacement of reactants from adsorption, alteration of viscosity, etc.) would appear to be involved; but the phenomenon requires further investigation.

Activation in mixtures of sea water and isotonic dextrose solution.—Acids have a special relation to activation in the starfish egg, as we have seen; and since the same sequence of physiological effects is produced by heat activation as by acid activation, the former is, in all likelihood, a special case of acid activation. In many cells acids are formed in large quantity as a result of moderate rise of temperature—for example, in the heat rigor of muscle; the typical source of the stronger acids formed under such conditions, as well as in normal metabolism (chiefly lactic, pyruvic, and other oxy-acids), is the breakdown of carbohydrate (glycolysis). Empirically, we find that the activation of starfish eggs in 0.004 M butyric acid in sea water at 20° proceeds at very nearly the same rate as heat activation in normal sea water at 32°; in both cases the optimal exposures lie typically between 6 and 9 minutes. The most noticeable difference is that in heat activation there is a distinct lag in membrane formation, which usually requires about 3 minutes' exposure to 32°, while in activation with 0.004 M butyric acid at room temperature the same effect is produced in 1 minute or less. This difference probably represents the time required for the metabolically produced acid to reach an effective concentration. According to the present hypothesis, the acid is regarded as producing its activating effect by setting free (e.g., by catalysis in some hydrolysis reaction) one of the reactants (*B*) of the activation reaction. The acid appears to act at a rate which is closely proportional to the increase of H-ion concentration within the egg above a certain level, since it is found that solutions of different penetrating acids which activate at the same rate (at constant temperature) have approximately the same cH values (R. S. Lillie, 1927).

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[6] Pincus and Shapiro (1940) found that rabbits' eggs could be activated very effectively by cooling (20° *in vitro*, or surrounding the Fallopian tube with a cold-water jacket).

Since carbohydrate readily gives rise to acid in metabolism, it might be expected, if activation is, in fact, associated with metabolic acid production (cf. p. 260), that artificial increase in the carbohydrate content of the egg would increase the rate of activation. by an additive kind of effect. The presence of dextrose in the medium in high concentration is nontoxic; the eggs undergo maturation, fertilization, and development to larval stages in mixtures of equal volumes or more of isotonic dextrose solution with sea water. Experimentally, it was found that if eggs are activated by acid dissolved in such mixtures, the rate of activation is markedly increased. Previous exposure to the neutral dextrose sea-water mixture is unimportant; the acid and dextrose must be present simultaneously in the medium. This is shown in the experiment described in Table 12. The rate of activation is seen to be approximately doubled in the dextrose sea-water mixture, and the same acceleration is shown whether the eggs have previously been exposed to the dextrose solution (*C*) or not (*D*). But eggs previously exposed to dextrose sea water and then activated by acid in sea water (*B*) show the same rate of activation as the control. This accelerating influence of dextrose was shown without exception in 10 similar series of experiments in the summer of 1940. The absence of any effect from the previous exposure to dextrose indicates nonpenetration (or slow penetration) of this substance into the resting egg. Activation is apparently associated with increased permeability to dextrose.

The degree of the acceleration increases with the concentration of dextrose (up to 75 vols. per cent). Table 13 shows this relation (one of three experiments giving the same result). The eggs were transferred directly from sea water to the dextrose–sea-water mixtures containing the acid.

Experiments were also performed with pure isotonic dextrose solution; but in such solutions the eggs die rapidly, because of the absence of salts, and no activation is possible. Fertilization in this solution is also impossible.

Experiments on heat activation in dextrose–sea-water mixtures have been few so far, but in two experiments in which the control eggs exposed to sea water at 32° yielded a fair proportion of blastulae (40–50 per cent with 6 minutes' exposure), eggs warmed to

32° in 50 volumes per cent and 75 volumes per cent dextrose sea water gave almost the same result, except for interference with membrane separation. This relative absence of effect would indicate that acceleration under these conditions, if present, is less than in the case of acid activation, but further experimentation is needed.

TABLE 12

June 19, 1940. Eggs from several starfish were used. Maturation and fertilization were normal in the great majority.

	Treatment	Result
A.	(Control.) 0.004 M butyric acid in sea water. Temperature 21° C.	Optimum at 6 min. with 80–85 per cent blastulae (7 min. slightly less favorable)
B.	Eggs kept for 22 min. in 75 vols. per cent dextrose–sea-water solution (75 vols. M-dextrose *plus* 25 vols. sea water); then to 0.004 M butyric acid in sea water	Similar to *A;* optimum at 6–7 min. (70–80 per cent blastulae)
C.	Eggs in 75 vols. per cent dextrose–sea-water solution as in *B;* then to 0.004 M butyric acid in the same solution	Optimum at 3–4 min. (65–75 blastulae)
D.	Eggs transferred directly from sea water to 0.004 M butyric acid in 75 vols. per cent dextrose–sea-water solution	Optimum at 3 minutes with 65–75 per cent blastulae; somewhat fewer blastulae at 4 min. (*ca.* 50 per cent)

TABLE 13

June 25, 1940. Eggs chiefly from one starfish. Maturation, fertilization and development are normal in most eggs.

	Treatment	Result
A.	(Control.) 0.004 M butyric acid in sea water. Temperature 17°–18° C.	Longest exposure (12 min.) is below optimum at this low temperture, and blastulae are few
B.	0.004 M butyric acid in 25 vols. per cent dextrose–sea-water solution	Optimum at 11 min. (longest exposure of series) with 50–60 per cent blastulae
C.	0.004 M butyric acid in 50 vols. per cent dextrose–sea-water solution	Optimum at 9–10 min. with 70–80 per cent blastulae
D.	0.004 M butyric acid in 75 vols. per cent dextrose–sea-water solution	Optimum at 8–9 min. with 70–80 per cent blastulae

In the experiments just described the eggs were exposed to dextrose sea water and activated during the prematurational period. Under normal conditions in sea water the response to artificial activation, as well as to sperm fertilization, falls off rapidly after the polar bodies are separated (R. S. Lillie, 1908); few, if any, eggs form blastulae if exposed to heat or acid after maturation is completed. If, however, the eggs are kept in dextrose–sea-water mixtures, instead of in sea water, for several hours after removal, they are found to retain, to a remarkable degree, the response to acid activation. This effect is illustrated in Table 14. Under these conditions, there would appear to be some intake of dextrose by the eggs, in contrast to the prematurational eggs. Maturation proceeds normally in such mixtures. A similar, though less favorable, result was obtained in experiments with 75 volumes per cent of dextrose sea water. Experiments of this kind with heat activation have not yet been tried.

TABLE 14

June 28, 1940. Eggs from one starfish; normal maturation (>90 per cent) and normal development after fertilization.

Treatment	Result
A. (Control.) Eggs exposed (during prematurational period) to 0.004 M butyric acid in sea water (19° C.)	Good activation with optimum at 10 min. (90 per cent blastulae)
B. Eggs in sea water for 35 min. after removal; then to 50 vols. per cent dextrose–sea-water solution for 3 hr. 11 min.; then to 0.004 M butyric acid in sea water	A large proportion (from a third to a half of the eggs) form blastulae at all exposures from 2 min. to 11 min. No well-defined optimum
C. Similar treatment with dextrose–sea-water solution, but eggs exposed to 0.004 M butyric acid in 50 vols. per cent dextrose–sea-water solution	Result similar to that in *B*, but the proportion of blastulae falls off at 8 min. None at 11 min.
D. Eggs left in sea water until time of activation (total stay in sea water *ca.* 4 hr.); then to 0.004 M butyric acid in 50 vols. per cent dextrose–sea-water solution	Blastulae numerous, but fewer than in *B* or *C*, with optimum at 6 min. (20–25 per cent). Few or none with exposures of 7–11 min.

A natural inference is that the normal loss of activability in the starfish egg after maturation results from depletion of carbohydrate. It is possible, however, that other factors may enter, such as the effect of decrease in the external salt concentration per se. According to Schechter (1937; *cf.* also Lindahl, 1936, p. 243; 1938), decrease in calcium greatly prolongs the fertilizable life of *Arbacia* eggs; and I have myself found that unfertilized *Arbacia* eggs live much longer in dextrose–sea-water mixtures than in pure sea water. The part played by concentration of salts, as such, requires further investigation.

CHEMICAL AND OTHER FACTORS IN ACTIVATION: GENERAL

The problem of the nature of the physicochemical, metabolic, and physiological factors which transform the egg from its inert or resting state into an active cell which automatically divides and passes through a complex cycle of development is a baffling one.[7] As already indicated, this problem forms part of the problem of the initiation of growth and differentiation in resting cells or tissues in general. The statement that activation removes a block to further activity merely expresses the problem in another form, i.e., it recognizes the pre-existence of latent or potentially active factors; the question is what releases these from quiescence—or "activates" them. To say that the course of metabolism is altered is a truism. There are evident parallels between the activation of egg cells and the various other biological manifestations of stimulation and response; but in making this comparison, we are doing no more than emphasizing the importance of fundamental conditions common to all reactive living systems. The methods of artificial parthenogenesis are described as nonspecific; in a somewhat similar sense the response of a nerve or muscle to stimulation is nonspecific; but under some conditions such a response may become specific, as in certain kinds of sensitization (e.g., anaphylactic). Somewhat analogously the starfish egg is nonspecific in its response to parthenogenetic agents, but it is also activated specifically by the sperm of its own species.[8] In the sequence of interlocked physicochemical events which constitute the activation process, it seems necessary to distinguish between certain primary, nonspecific physical events which initiate metabolic reactions of a simple type (e.g., by some kind of surface effect) and later more complex events, following upon the first, into which specific metabolic reactions enter. Such specific reactions may occur at an early stage in fertilization, as seen in the fertilizin reaction of the sea-urchin egg (F. R. Lillie, 1919). The problem of artificial parthenogenesis has reference more particularly to the nature of the initial nonspecific changes, both physical and chemical. The special metabolic and morphogenetic cycle which follows is uniquely characteristic for each species; it may be assumed that each developmental stage has its own special physiological and biochemical features and that the transition to the physiology of the adult organism is gradual rather than abrupt.

On the physical side the nonspecific reaction of activation appears very generally to be associated with a structural alteration of the surface layer or cortical zone of the egg. This is shown with unusual clearness in certain eggs, e.g., the *Nereis* egg (F. R. Lillie, 1912, 1919). The majority of studies have been made with the echinoderm egg; on its first contact the spermatozoon initiates locally a reaction which then spreads rapidly over the whole egg surface (Just, 1922, 1939). One of its manifestations in echinoderm eggs (and some others) is the separation of the fertilization membrane. There appears to be a temporary structural breakdown (of the kind which, if permanent, leads to cytolysis) of the most superficial layer of protoplasm; and this change is, in many cases, associated with an increased permeability to water-soluble substances of low molecular weight and water. This is notably the case in the sea-urchin egg, but in the starfish egg there appears to be no immediate change of permeability on fertilization (R. S. Lillie, 1918, p. 420). In the sea-urchin egg the associated changes of electrical conductivity have been measured by McClendon (1910) and Gray (1913). The connection between the increase of permeability and the metabolic and other immediate effects of activation has been variously interpreted. Substances formerly kept apart by diffusion-proof partitions (such as alveolar walls) may become free to interact when these barriers become permeable, and the resulting reaction may form the initial metabolic step in activation. The experiments described above show how the rate of the activation reaction may be influenced by changing the physical density (as determined by water content) of the surface layer of the egg. Other reactions may also be accelerated or enabled to occur by the structural changes of activation. The characteristic metabolic sequence determined by the special organization of the egg may thus be set in motion. Another general physical consequence of increased permeability is a change in the electrical potential difference across the cell surface, and it is possible that such variation of potential (analogous to the action potential of stimulation) may in itself be a critical factor in activation. The facts of electrical parthenogenesis show that the activation reaction may be released by changes of electrical polarization; and the mode of travel of the initial membrane-forming surface reaction (cf. Just, 1922; Whitaker, 1931) suggests its dependence on the formation of local electrical circuits, as in other cases of protoplasmic transmission. A transmitted electrical variation in the frog's egg activated by puncture has recently been demonstrated by Rothschild and Péterfi (1935). Structural changes in the cortical regions of various eggs following activation have been described by many investigators, using a variety of methods. (Some recent papers, giving references to the older literature, are: Runnström, 1928; Hobson, 1932; Chambers, 1933; Moser, 1939; Novikoff, 1939.)

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[7] Cf. the recent interesting discussion of Dalcq, Pasteels, and Brachet (1936).

[8] Loeb tried to abolish this specificity, or block to fertilization by foreign sperm, and to some extent was able to do so in sea-urchin eggs by making the sea water hyperalkaline (cf. J. Loeb, 1913*a*, p. 225).

The general view that two processes, physically and chemically distinct, are concerned in activation—(1) a process localized in the surface layer of the egg and associated with a structural change of a cytolytic kind ("lysin theory") and (2) a more internally situated metabolic process involving synthesis—was early supported by Loeb (cf. Loeb, 1913*a*, chap. xii). One of his conclusions was that the critical metabolic change in activation is an increase in certain intracellular oxidations. Increased oxygen consumption in the sea-urchin egg after fertilization was demonstrated by Warburg in 1908 and by Loeb and Wasteneys a little later; and artificial forms of activation (hypertonic sea water, fatty acid) were found to have the same effect.[9] But it has since been found that such a change is by no means general; fertilization may leave respiration unchanged or may even decrease it in some cases, as in the eggs of *Chaetopterus* and *Cumingia*, studied by Whitaker (1933). Even in eggs where fertilization increases respiration, the effect may vary greatly at different temperatures (Gerard and Rubenstein, 1934). Nevertheless, a change in the conditions controlling oxygen consumption appears to be an important feature of activation in certain cases. In the unfertilized sea-urchin egg respiration is low and is almost unaffected by cyanide; but on fertilization it increases several times and cyanide-sensitive factors enter—a fact (among others) indicating that new catalyzing systems come into operation at that time (Runnström, 1930; Korr, 1937; Lindahl, 1938, 1939). A similar increase in respiration can be induced in the unfertilized egg by methylene blue, *p*-phenylene diamine, or pyocyanin (Barron, 1929; Örström, 1932; Runnström, 1930, 1932*a*, 1935*a*), or by nitrophenols (Clowes and Krahl, 1936), but without activating the egg or removing its capacity for fertilization. Increase in respiration is thus not in itself sufficient for activation, although it may be associated with activation as one of its direct or indirect consequences.

Another metabolic effect of activation, which may have a more general importance, is the production of acid; this effect is probably localized in the surface layer of the egg. A temporary release of acid, sufficient to displace considerable quantities of CO_2, was shown by Runnström (1933) to occur in the sea-urchin egg during the period immediately following fertilization; and more recent studies by Rothschild (Laser and Rothschild, 1939) indicate that this special acid-producing reaction is completed within a brief period of about 5 minutes. The brevity of this initial reaction is of interest in relation to the fact that in the starfish egg the activating effect of external acid may be completed within an equally short or shorter time. The question as to the nature and source of the acid formed in fertilization is still unanswered; it is also uncertain how far this phenomenon is general and not a special peculiarity of the sea-urchin egg. When we consider the general features of cellular metabolism, it seems natural to refer the acid to carbohydrate metabolism. Fertilization and artificial activation occur readily under anaerobic conditions, as does also the metabolic production of lactic and other acids in many cells. Various attempts have been made, especially by Runnström and his associates (cf. Lindahl's review, 1938), to correlate acid production in eggs with the same phenomenon in yeast, muscle, and other cells. The acid formed in activation is unidentified; lactic acid seems to be excluded by the absence of interference by iodoacetate and fluoride (Runnström, 1935*b* and *c;* R. S. Lillie, 1934); the same conclusion would hold for other acids which arise in the usual types of glycolytic breakdown (e.g., pyruvic), although this may not be certain (cf. Rothschild, 1939). Although lactic acid has been demonstrated in small quantity in both fertilized and unfertilized sea-urchin eggs, there appears to be little change on fertilization (cf. Perlzweig and Barron, 1928). Some rapidly diffusing acid would seem to be indicated by the parallels between heat activation and acid activation in starfish eggs, if we assume that the acid produced in heat activation is the same as in normal fertilization. Heat activation can be arrested at any stage by transferring the eggs to normal sea water at 20°, just as can acid activation. The view that the acid of activation is a lower fatty acid derived from fat metabolism has recently been supported by Hayes (1938), who finds a decrease in the fat content of sea-urchin eggs after fertilization, and he suggests a partial transformation of fat into lower fatty acid.

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[9] Cf. Needham, 1931, 2:623 ff., for an account of this earlier work.

There are difficulties in regarding acid production as the initiatory metabolic event in all cases of activation. Ammonia is a very efficient activating agent in certain eggs; the *Urechis* egg, recently studied by Tyler, is a good instance (Tyler and Bauer, 1937; Tyler and Horowitz, 1938); Loeb also found various organic bases to be effective in the sea-urchin egg (J. Loeb, 1913*a*, p. 147). In the starfish egg, where the relation of acid to activation is especially clear, ammonia has no activating effect (R. S. Lillie, 1914). Yet the possibility remains that ammonia, because of its special importance in nitrogen metabolism, may form an essential link in the chain of activating reactions. Ammonia penetrates cells readily, and its addition from without might furnish the factor needed to complete activation in some cases. The relation of ammonia metabolism to activation in the sea-urchin egg has recently been investigated by Örström (1937), who finds a marked increase in the assimilation of ammonia after fertilization in the presence of oxygen; this effect is associated with nuclear changes, and he calls attention to possibly significant analogies with ammonia metabolism in the vertebrate liver. In cases where both acid and ammonia may have activating effects, e.g., the *Urechis* egg (Tyler and Bauer, 1937), it would seem that their respective places in the system of activating reactions must be entirely different.

In an earlier paper (R. S. Lillie, 1931), I made the tentative suggestion that a splitting of phosphate esters might form one of the steps in activation, having in mind especially the analogy of muscle metabolism. This would release phosphate from combination; there is, however, no evidence of an increase in free phosphate in the egg during activation, although this has been looked for (Runnström, 1932*b*; R. S. Lillie, 1934). It should be noted, however, that if the phosphate is not set free within the egg but is simply transferred from one form of ester combination to another, an effect equivalent to acid production might result. The formation of hexose-phosphoric acid, which is a stronger acid than orthophosphoric (cf. Meyerhof, 1930, p. 74; 1937, p. 21), might conceivably be a factor in the observed increase in acidity. This view is consistent with the above described effects of dextrose–sea-water mixtures.[10] A sufficient increase of acidity, in whatever way effected, at the site of the activation reaction (cortical zone) appears to have an activating effect on the starfish egg; in my own comparative study (1927) of the activating efficiency of different acids a constant rate of activation was found to be associated with a nearly constant increase of cH.

Granting the importance of acid, it remains to be determined just how increase of cH can alter the state of the resting egg in such a way as to cause activation. Loeb had suggested as early as 1907 (J. Loeb, 1907) that the primary chemical change underlying membrane formation was some simple splitting process—for example, hydrolysis—and the view that a reaction of this general type may form the first chemical step in activation has been favored by Runnström (1932*b*, 1939) and others. Some compound thus released is regarded as entering directly or indirectly into other reactions of a synthetic kind. Such a conception is in agreement with the general evidence, considered above, that two distinct types of reaction product unite to form a third compound (the "activating substance") the accumulation of which brings the egg into the automatically developing state (R. S. Lillie, 1931, 1934). According to this general view, the activating substance would play a role physiologically analogous to that of a growth-determining hormone.

The problem of the nature of the compound (symbolized as *B* in the scheme) which is progressively released in the egg cortex by increase of acidity is one on which only conjecture is possible at present. Some compound whose diffusion rate is readily affected by changes in the concentration of the protoplasmic gel would seem to be indicated by the experiments with dilute and concentrated sea water described above. Such a molecule must be relatively large; fatty acid diffuses readily into and out of the egg, but the compound formed under its influence is not lost when the egg is transferred to sea water but remains within the system, having apparently entered into some new combination. This is indicated by such facts as partial activation and the cumulative effects of two or more successive treatments with heat or acid. The splitting of a phosphagen such as arginin phosphate would release arginin, the physical properties of which would perhaps answer to the requirements, while as an amino acid this compound might be important in synthesis; but any such identification is purely speculative at present.

The formation of the activating compound *A* (granting it to be formed) represents only a stage or necessary step in the sequence of metabolic events determining development. The initiation of cell division involves changes in both nuclear and cytoplasmic metabolism. Brachet (1938) has demonstrated an increase in thymo-nucleic acid in artificially activated sea-urchin eggs; this increase is similar to that occurring in fertilized eggs but more gradual. There may be a relation here to the changes in nitrogen metabolism described by Örström, since protein metabolism is fundamental in all processes of growth and development. That activation is associated with a change in the physical state of the structural proteins is indicated by the variations observed by Heilbrunn and others in the consistency or viscosity of the egg protoplasm following fertilization (Heilbrunn, 1915, 1920, 1937); the increase in free calcium described by Heilbrunn (1937, cf. pp. 538, 544) and Mazia (1937) may have its importance here. Recently Mirsky (1936) has found an increase in the water-soluble proteins of sea-urchin eggs after fertilization.

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[10] However, the phosphorylation of hexoses is inhibited by iodoacetate (as pointed out by Runnström, 1935*b*), which does not prevent activation.

It is not surprising that the metabolism of artificially activated eggs should agree closely with that of fertilized eggs, since, if activation is complete, both kinds must pass through the same cycle of transformation; and the experiments of Brachet (1938) and others indicate that, as regards oxygen-consumption and other metabolic changes following fertilization, there is no essential difference. Tyler and Horowitz (1938) also find that the initial rate of respiration is the same in the artificially activated as in the fertilized eggs of *Urechis*. As development advances, divergences must be expected, since it is only exceptionally that artificially activated eggs develop without deficiencies of some kind. In this brief review, however, we are concerned only with the nature of the initiating process. The broader problem regarding the nature of the factors which determine and integrate the highly special processes forming the later parts of the developmental sequence is beyond the scope of our present discussion.

SUMMARY

1. Unfertilized starfish eggs, after an exposure to hypertonic sea water of 10–30 minutes, followed by return to normal sea water, show an increased rate of activation by acid (e.g., 0.004 M butyric acid in sea water) or heat (e.g., sea water at 32°). This "sensitizing" effect of hypertonic sea water increases (within certain limits) with concentration and with the duration of exposure.

2. If, however, the eggs are subjected to these activating conditions while they are still in a water-depleted state—for example, if they are transferred directly from the hypertonic sea water to a solution of acid in the same medium—the rate of activation is found to be decreased, to a degree depending directly on the concentration of the hypertonic medium.

3. In these experiments (under 2) the duration of the previous exposure to hypertonic sea water (between 1 minute and 30 minutes) makes little difference in the rate of activation in the acid-containing medium. Since the surface layer (cortical region) of the egg is the only region that can be regarded as reaching a nearly constant water content within so brief a period as one minute, this independence of time is evidence that the site of the activating reaction is superficial, i.e., in the cortical layer of the egg.

4. Similar experiments with dilute sea water (up to 60 volumes per cent) lead to the same conclusion. Temporary exposure of eggs to dilute sea water has, in itself, no activating effect. On the other hand, the immediate influence of dilute sea water on the rate of activation is definite and is opposite in direction to that of hypertonic sea water; i.e., in eggs immersed in dilute sea water, with or without previous exposure to the same medium, the activation reaction is accelerated, to a degree increasing with dilution; this effect is also independent of the duration of the previous exposure.

5. The velocity of the activation reaction thus appears dependent on the water content (structural density) of the cortical region, increasing as the water content increases. This result is interpreted as indicating that the reaction velocity is determined (in part) by the rate of diffusion of reactants in this region, which would appear to have the physical properties of a somewhat dense gel.

6. In mixtures of sea water with isotonic dextrose solution the rate of activation by acid is greater than in normal sea water and increases with the concentration of dextrose (up to 75 volumes per cent dextrose solution).

7. The loss of activability shown normally by starfish eggs kept in sea water beyond the maturation period is delayed or prevented if the eggs are kept in dextrose–sea-water mixtures. (Maturation proceeds normally in such mixtures).

8. A brief general discussion of the physicochemical and metabolic factors of activation is given.

LITERATURE CITED

Barron, E. S. G. 1929. The effect of methylene blue on the oxygen consumption of the eggs of the sea-urchin and starfish. Jour. Biol. Chem., 81:445.

Bodine, J. H. 1932. Hibernation and diapause in certain Orthoptera. Physiol. Zoöl., 5:538 and 549.

Bodine, J. H., and Boell, E. J. 1934. Respiratory mechanisms of normally developing and blocked embryonic cells. Jour. Cell. and Comp. Physiol., 5:47.

Brachet, J. 1938. Oxygen consumption of artificially activated and fertilized *Chaetopterus* eggs. Biol. Bull., 74:93.

Chambers, R. 1921*a*. Studies in the organization of the starfish egg. Jour. Gen. Physiol., 4:41.

———. 1921*b*. Microdissection studies. III. Some problems in the maturation and fertilization of the echinoderm egg. Biol. Bull., 41:318.

———. 1933. The manner of sperm entry in various marine ova. Jour. Exper. Biol., 10:130.

Child, C. M. 1940. Lithium and echinoderm exogastrulation: with a review of the physiological gradient concept. Physiol. Zoöl., 13:4.

Clowes, G. H. A., and Krahl, M. E. 1936. Studies on cell metabolism and cell division. I and II. Jour. Gen. Physiol., 20:145 and 173.

———. 1940. Studies on cell metabolism, etc. IV. *Ibid.*, 23:413.

Dalcq, A., Pasteels, J., and Brachet, J. 1936. Données nouvelles (*Asterias glacialis*, *Phascolion strombi*, *Rana fusca*) et considérations théoriques sur l'inertie de l'œuf vierge. Mém. d. Mus. roy. hist. nat. Belgique, 2me sér., fasc. 3, p. 881.

Delage, Y. 1901. Etudes expérimentales sur la maturation cytoplasmique et sur la parthénogénèse chez les échinodermes. Arch. zool. expér. et générale, 3me sér., 9:285.

Freundlich, H. 1923. Colloid and capillary chemistry. New York: Dutton & Co.

Gerard, R. W., and Rubenstein, B. B. 1934. Fertilization and the temperature coefficients of oxygen consumption in eggs of *Arbacia punctulata*. Jour. Gen. Physiol., 17:677.

Goddard, D. R. 1935. The reversible heat activation inducing germination and increased respiration in the ascospores of *Neurospora tetrasperma*. Jour. Gen. Physiol., 19:45.

Godlewski, E. 1913. Physiologie der Zeugung. In Winterstein's Handb. d. vergl. Physiol., 3: Part II, 805–67.

Gray, J. 1913. The electrical conductivity of fertilized and unfertilized eggs. Jour. Mar. Biol. Assoc., 10:50.

Greeley, A. W. 1902. Artificial parthenogenesis in starfish eggs produced by lowering of temperature. Amer. Jour. Physiol., 6:296.

Harvey, E. B. 1936. Parthenogenetic merogony or cleavage without nuclei in *Arbacia punctulata*. Biol. Bull., 71:101.

———. 1940. A comparison of the development of nucleate and non-nucleate eggs of *Arbacia punctulata*. *Ibid.*, 79:166.

Harvey, E. N. 1910. Methods of artificial parthenogenesis. Biol. Bull., 18:269.

Hayes, F. R. 1938. The relation of fat changes to the general chemical embryology of the sea-urchin. Biol. Bull., 74:267.

Heilbrunn, V. 1915. Studies in artificial parthenogenesis. II. Physical changes in the egg of *Arbacia*. Biol. Bull., 29:149.

———. 1920. An experimental study of cell-division. I. Jour. Exper. Zoöl., 30:211.

———. 1937. An outline of general physiology. Philadelphia: W. B. Saunders Co.

Hobson, A. D. 1932. The effect of fertilization on the permeability to water and on other properties of the surface of the egg of *Psammechinus miliaris*. Jour. Exper. Biol., 9:69.

JUST, E. E. 1922*a*. The effect of hypertonic sea-water in producing membrane separation, cleavage and top-swimming plutei. Biol. Bull., 43:384.

———. 1922*b*. The fertilization reaction in *Echinarachinius parma*. Amer. Jour. Physiol., 61:516.

———. 1939. The biology of the cell surface. Philadelphia: Blakiston.

KORR, I. 1937. Respiratory mechanisms in the unfertilized and fertilized sea-urchin egg: A temperature analysis. Jour. Cell. and Comp. Physiol., 10:461.

KRAEMER, E. O. 1931. Colloids. Chap. xx in H. S. Taylor (ed.), Treatise on physical chemistry. New York: van Nostrand Co.

LASER, H., and ROTHSCHILD, LORD. 1939. The metabolism of the egg of *Psammechinus miliaris* during the fertilization reaction. Proc. Roy. Soc., London B., 126:539.

LILLIE, F. R. 1902. Differentiation without cleavage in the egg of the annelid *Chaetopterus pergamentaceus*. Arch. f. Entw'mech., 14:477.

———. 1912. Studies of fertilization in *Nereis*. III. Jour. Exper. Zoöl., 12:413.

———. 1919. Problems of fertilization. Chicago: University of Chicago Press.

LILLIE, R. S. 1908. Momentary elevation of temperature as a means of producing artificial parthenogenesis in starfish eggs. Jour. Exper. Zoöl., 5:375.

———. 1910. The action of isotonic solution of neutral salts on the unfertilized eggs of *Asterias* and *Arbacia*. Amer. Jour. Physiol., 26:106.

———. 1911. The action of calcium salts, etc. *Ibid.*, 27:289.

———. 1914. Mass action in the activation of unfertilized starfish eggs by butyric acid. Jour. Biol. Chem., 24:233.

———. 1915. On the conditions of activation of unfertilized starfish eggs under the influence of high temperature and fatty acid solution. Biol. Bull., 28:260.

———. 1917. Temperature coefficients in the activation of starfish eggs by butyric acid. *Ibid.*, 32:131.

———. 1918. The increase in permeability to water in fertilized sea-urchin eggs and the influence of cyanide and anaesthetics on this change. Amer. Jour. Physiol., 45:406.

———. 1926, 1927. The activation of starfish eggs by acid. I, II. Jour. Gen. Physiol., 8:339; 10:703.

———. 1931. The influence of cyanide and lack of oxygen on the activation of starfish eggs by acid, heat and hypertonic sea-water. Biol. Bull., 60:288.

———. 1934. The influence of hypertonic and hypotonic sea-water on the artificial activation of starfish eggs. *Ibid.*, 66:361.

LILLIE, R. S., and BASKERVILL, M. L. 1922. The action of ultraviolet rays on starfish eggs. Amer. Jour. Physiol., 61:57.

LILLIE, R. S., and CATTELL, W. 1925. The conditions of activation of unfertilized starfish eggs by the electric current. Biol. Bull., 49:100.

LILLIE, R. S., and HINRICHS, M. A. 1923. Activation of starfish and sea-urchin eggs by photodynamic action. Anat. Rec., Suppl., 26:370.

LINDAHL, E. 1936. Zur Kenntnis der physiologischen Grundlagen der Determination im Seeigelkeim. Acta zool., 17:180.

———. 1938. Physiologisch-chemische Probleme der Entwicklung. Fortschr. d. Zool., N.F., 3:271.

———. 1939. Ueber die biologische Sauerstoffaktivierung nach Versuchen mit Kohlenmonoxyd an Seeigeleiern und Keimen. Zeitschr. f. vergl. Physiol., 27:138.

LOEB, J. 1899. On the nature of the process of fertilization and the artificial production of normal larvae (plutei) from the unfertilized eggs of the sea-urchin. Amer. Jour. Physiol., 3:135.

———. 1907. The chemical character of the process of fertilization and its bearing upon the theory of life phenomena. Univ. of California Publ., Physiology, 3:61.

———. 1913*a*. Artificial parthenogenesis and fertilization. Chicago: University of Chicago Press.

———. 1913*b*. Further experiments on natural death and prolongation of life in the egg. Jour. Exper. Zoöl., 15:201.

———. 1920. Further observations on the production of parthenogenetic frogs. Jour. Gen. Physiol., 3:539.

LOEB, J., and BANCROFT, F. W. 1913. The sex of a parthenogenetic tadpole and frog. Jour. Exper. Zoöl., 14:275.

LOEB, LEO. 1911. The parthenogenetic development of ova in the mammalian ovary and the origin of ovarian teratomata and chorioepitheliomata. Jour. Amer. Med. Assoc., 56:1327.

———. 1915. Spontaneous parthenogenesis of the eggs in the ovary of the guinea pig. Biol. Bull., 28:59.

Loeb, Leo. 1920. Causes and definitions of cancer. Amer. Jour. Med. Sci., 159:781.

McClendon, J. F. 1910. Changes in permeability of developing eggs to electrolytes. Amer. Jour. Physiol., 27:240.

Mathews, A. P. 1901. Artificial parthenogenesis by mechanical agitation. Amer. Jour. Physiol., 6:142.

Mazia, D. 1937. The release of calcium in *Arbacia* eggs on fertilization. Jour. Cell. and Comp. Physiol., 10:291.

Meyerhof, O. 1930. Die chemischen Vorgänge im Muskel. Berlin: J. Springer.

———. 1937. Ueber die Intermediarvorgänge der enzymatischen Kohlehydratspaltung. Ergebn. d. Physiol., 39:10.

Mirsky, A. E. 1936. Protein coagulation as a result of fertilization. Science, 84:333.

Moser, F. 1939. Studies in a cortical layer response to stimulating agents in the *Arbacia* egg. Jour. Exper. Zoöl., 80:423.

Needham, J. 1931. Chemical embryology. 3 vols. New York: Macmillan.

Novikoff, A. B. 1939. Changes at the surface of *Nereis limbata* eggs after insemination. Jour. Exper. Biol., 16:403.

Örström, A. 1932. Zur Analyse der Atmungsteigerung bei der Befruchtung des Seeigeleies u.s.w. Protoplasma, 15:566.

———. 1937. Ueber den Ammoniakstoffwechsel im Seeigelei. Naturwiss., 25:300.

Owen, S. E. 1937. Sex hormones, carcinogenics and sterols. Quart. Rev. Biol., 12:340.

Parmenter, C. L. 1925. The chromosomes of parthenogenetic frogs and tadpoles. Jour. Gen. Physiol., 8:1.

———. 1940. Chromosomes in *Rana fusca* parthenogenetically developed from eggs with known polar body and cleavage histories. Jour. Morph., 66:241.

Perlzweig, W. A., and Barron, E. S. G. 1928. Lactic acid and carbohydrate in sea-urchin eggs under aërobic and anaërobic conditions. Jour. Biol. Chem., 79:19.

Pincus, G. 1939. The comparative behavior of mammalian eggs *in vivo* and *in vitro*. IV. The development of fertilized and artificially activated eggs. Jour. Exper. Zoöl., 82:85.

Pincus, G., and Shapiro, H. 1940. The comparative behavior of mammalian eggs *in vivo* and *in vitro*. VII. Further studies on the activation of rabbit eggs. Proc. Amer. Philos. Soc., 83:631.

Rothschild, Lord. 1939. The effect of phlorizin on the metabolism of cytolysing sea-urchin eggs. Jour. Exper. Biol., 16:49.

Rothschild, V., and Péterfi, J. 1935. Bioelectric transients during fertilization. Nature, 135:874.

Rugh, R. 1937. A quantitative analysis of the pituitary-ovulation relations in the frog (*Rana pipiens*). Physiol. Zoöl., 10:84.

Runnström, J. 1928. Die Veränderungen der Plasmakolloide bei der Entwicklungserregung des Seeigeleies. Protoplasma, 4:388 and 5:201.

———. 1930. Atmungsmechanismus und Entwicklungserregung bei dem Seeigelei. *Ibid.*, 10:106.

———. 1932*a*. Die Beeinflussung der Atmung und der Spaltung in Seeigelei u.s.w. *Ibid.*, 15:532.

———. 1932*b*. Ueber den Mechanismus der Entwicklungserregung bei dem Seestern- und Seeigelei. *Ibid.*, 15:448.

———. 1933. Zur Kenntnis der Stoffwechselsvorgänge bei der Befruchtung des Seeigeleies. Biochem. Zeitschr., 258:257.

———. 1935*a*. On the influence of pyocyanine on the respiration of the sea urchin egg. Biol. Bull., 68:327.

———. 1935*b*. Acid formation in frozen and thawed *Arbacia* eggs and its possible bearing on the problem of activation. *Ibid.*, 69:345.

———. 1935*c*. Influence of iodoacetate on activation and development of the eggs of *Arbacia punctulata*. *Ibid.*, 69:351.

———. 1939. Ueber den Einfluss von Kupfer auf die Entwicklungserregung des Eies von *Arbacia*, nebst Bermerkungen über das Problem der Entwicklungserregung. Protoplasma, 32:366.

Schechter, V. 1937. Calcium reduction and the prolongation of life in the egg cells of *Arbacia punctulata*. Biol. Bull., 72:366.

Stewart, D. R., and Jacobs, M. H. 1932. The effect of fertilization on the permeability of the eggs of *Arbacia* and *Asterias* to ethylene glycol. Jour. Cell. and Comp. Physiol., 1:83.

Tyler, A. 1931*a*. The production of normal embryos by artificial parthenogenesis in the echiuroid *Urechis*. Biol. Bull., 60:187.

———. 1931*b*. The relation between cleavage and total activation in artificially activated eggs of *Urechis*. *Ibid.*, **61**:45.

———. 1932. Production of cleavage by suppression of the polar bodies in artificially activated eggs of *Urechis*. *Ibid.*, **63**:218.

TYLER, A., and BAUER, H. 1937. Polar body extrusion and cleavage in artificially activated eggs of *Urechis caupo*. Biol. Bull., **73**:164.

TYLER, A., and HOROWITZ, N. H. 1938. On the energetics of differentiation. VII. Comparison of the respiratory rates of parthenogenetic and fertilized *Urechis* eggs. *Ibid.*, **74**:99.

WEISS, P. 1939. Principles of development. New York: Henry Holt & Co.

WENT, F. W., and THIMANN, K. V. 1937. Phytohormones. New York: Macmillan.

WHITAKER, D. 1931. On the conduction of the cortical change at fertilization in the starfish egg. Biol. Bull., **60**:23.

———. 1933. On the rate of oxygen consumption by fertilized and unfertilized eggs. *Jour. Gen. Physiol.*, **16**:497.

WILSON, E. B. 1901. Experimental studies in cytology. I. A cytological study of artificial parthenogenesis in sea-urchin eggs. Arch. f. Entw'mech., **12**:529.

MITOCHONDRIAL ORIENTATIONS AND MOLECULAR PATTERNS

(Four text figures; one plate)

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THOSE who have studied mitochondria have frequently noted their rather specific orientations in cells where there were no obvious structural features of the cytoplasm, such as cell shape, densely crowded droplets, myofibrillae, etc., to account for such an arrangement. For instance, every textbook of histology states that the mitochondria of columnar epithelium have their long axes parallel with the principal axis of the cell. The author (Pollister, 1932) briefly suggested that these mitochondrial orientations are better described as parallel with the most direct course of diffusion through the cytoplasm. The most conclusive evidence of this is from the large hepatic cells of *Amphiuma* (Text Fig. 1, *A*). The often described orientation "parallel with the main axis of the cell" (Text Fig. 1, *B*) is strictly true only of those parts of the cytoplasm of a columnar cell that happen also to be in the course of diffusion. For above and below the nucleus, which is an obstruction to diffusion, mitochondria are oriented at random. The concentration gradients (Text Fig. 1, *D* and *E*) that determine diffusion through these epithelia are obvious; they are a consequence of the cell's being in a relation where two functional surfaces are in contact with two different fluids, while the other surfaces, the sides, are in contact with adjacent epithelial cells and hence are not functional. In an approximately spherical cell, like a leucocyte floating in blood plasma or tissue fluid (Text Fig. 1, *C*), the entire surface is in contact with the same medium, and there can be no concentration gradients and no diffusion directly across the whole cell. The gradients must, instead, be between the cell surface and the approximate center of the main cytoplasmic mass (Text Fig. 1, *F*); diffusion outward and inward must be along these gradients and hence radial to the approximate center of the main mass of cytoplasm. Mitochondria of the leucocyte are radial to this cytoplasmic center and so, just as in epithelia, parallel with the line of flow or diffusion.

In previous studies (Pollister, 1933*a*, 1933*b*, 1939) it has been noted that the physiological differences between what may be termed the "longitudinally polarized epithelial cell type" and the "centrally polarized leucocyte type" are accompanied by structural differences—in position of centrioles and shape of the Golgi apparatus. In the epithelial cell type the centrioles are at the lumen or distal end of the cell (Text Fig. 2, *A*); in the leucocyte these small granules are near the center of the main mass of cytoplasm (Text Fig. 2, *B*). The epithelial cell has a Golgi apparatus in the form of a vertical collar, so oriented as not to intercept the line of flow of materials through the cell (Text Fig. 2, *A*); in the leucocyte (and other centrally polarized cells, such as connective tissue, myoblasts, and neuroblasts) the Golgi apparatus is a horizontal collar, a perforated disk, likewise oriented to conform to the course of diffusion (Text Fig. 2, *B*).

The present report concludes this survey of the cytoplasmic constituents of the cells of Amphibia with a detailed consideration of orientations of mitochondria.

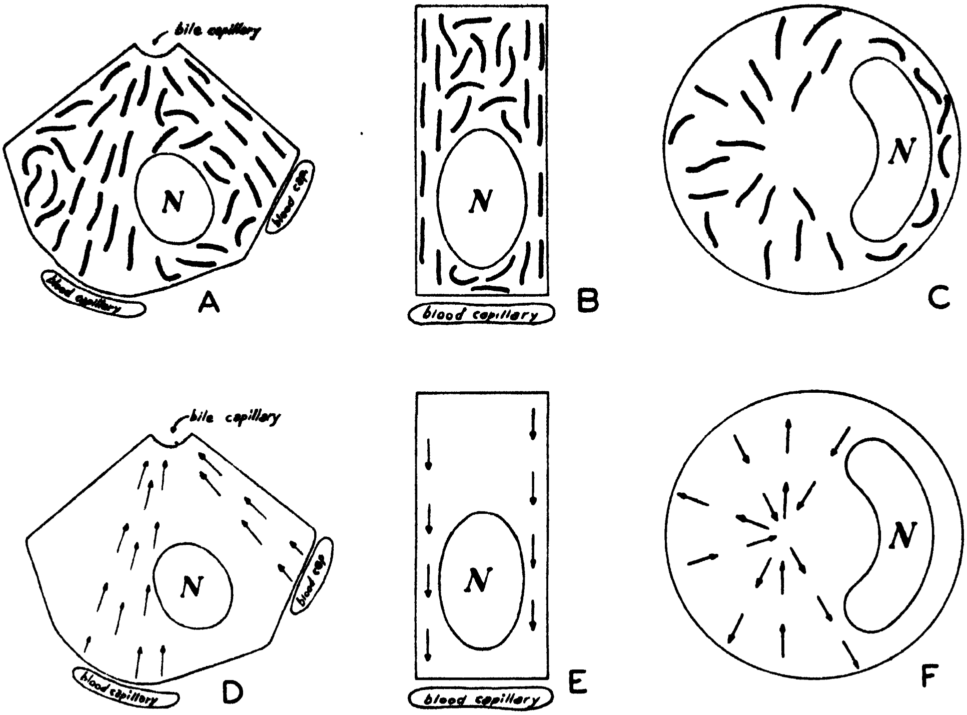

FIG. 1.—Schematic figures of orientations of mitochondria in relation to most direct course of diffusion in tissue cells. Upper row, mitochondria: *A*, hepatic epithelium, *Amphiuma; B*, typical columnar cell. from surface epithelium of intestine; *C*, spherical leucocyte. Lower row, most direct course of diffusion, shown by arrows: between blood capillary and lumen end of epithelial cells, *D*, *E;* and radial to center of main cytoplasmic mass in leucocyte (*F*), which is in contact with the same fluid over entire surface. *N*, nucleus.

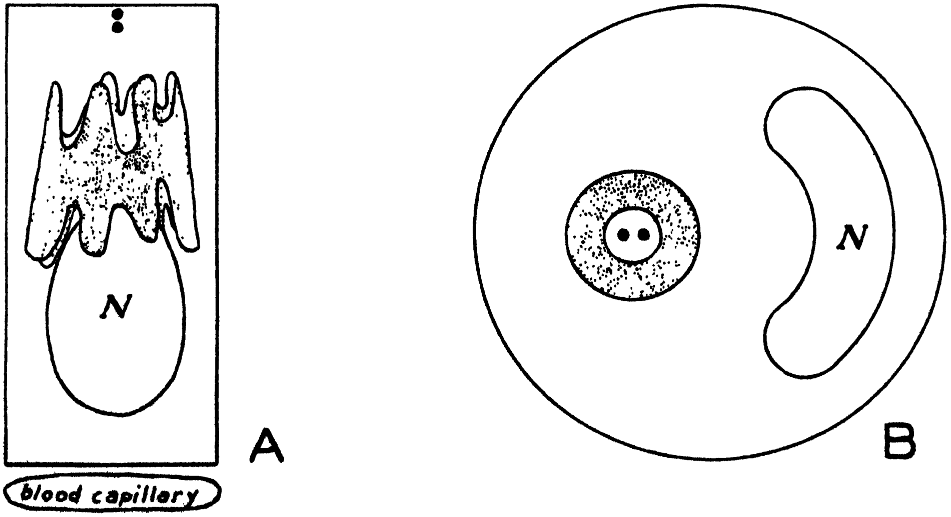

FIG. 2.—Schematic figures of Golgi apparatus, stippled, and centrioles, solid black, in epithelial cell type (*A*) and leucocyte type (*B*). *N*, nucleus.

MATERIAL AND METHODS

The material was all prepared by standard mitochondrial methods of either Benda or of Kull. One must be critical about mitochondrial fixation and must select those cells which contain only rodlike mitochondria of uniform diameter and of nearly uniform length, since the study of cells in tissue culture has made it evident that the granules and shorter filaments are artifacts in most cases, the notable exception being probably all egg cells. All cells are drawn from the tissues of urodelous Amphibia, in which are found the largest vertebrate cells—though by no means necessarily the largest mitochondria.

OBSERVATIONS

Here we are not interested in the mitochondria, per se, but only in their arrangements—as a clue to the organization of the optically structureless protoplasmic background, the hyaloplasm, which must be regarded as the real site of most of the important workaday activities of the cell. In cells that are filled with stored relatively inert material, as in a liver cell (Pl. I, Fig. 2) the hyaloplasm is crowded into interstices between these droplets; and the orientation of the mitochondria is, of course, likewise determined by their being forced into these narrow strands of hyaloplasm. There are other cells, like the fibroblast (Pl. I, Fig. 1), in which the entire cytoplasm is drawn out into long threadlike processes; and, obviously, in such a case the mitochondrial threads can merely be oriented parallel with these processes. In both these cell types the orientation of the mitochondria tells us nothing of the underlying cytoplasmic structure.

There are other cells, notably those of muscle, nerve, and epidermis, where the entire cytoplasmic mass is in a highly organized visible pattern of what are presumably the active functional elements—respectively myofibrillae, neurofibrillae, and tonofibrillae. In these cells the mitochondria are included in this pattern; and the study of their orientation in the fully differentiated cell can teach us nothing additional about the organization of the hyaloplasm. Examination of the mitochondria as these cells differentiate is, however, enormously instructive, for the progress in exact arrangement of these apparently passive rodlets gives us a reflection of the developing molecular organization that results in the differentiated fibrillae (see below, p. 276).

In other tissue cells we see the mitochondria against the typical hyaloplasmic background. When the cell is alive, or when it is as well fixed as it frequently is by the mitochondrial methods, the hyaloplasm appears structureless and homogeneous by ordinary microscopic examination. If there were no mitochondria in it, we should, in most cells, be forced to resort to conjecture as to the organization of this hyaloplasm; or, more likely, we would probably not suspect there was an organization. As it is, mitochondrial orientations give us a definite clue to the cytoplasmic organization.

In a typical leucocyte, a cell that is centrally polarized (Pl. I, Fig. 3), the long mitochondrial threads are oriented at random except where they are radial to a point at the approximate center of the main mass of cytoplasm. There is a similar orientation in the cartilage cell (Pl. I, Fig. 4). In the Champy-fixed material, from which Figures 3 and 4 of Plate I were drawn, there appears to be no structure in the hyaloplasm. But in material fixed in strong protein coagulants (Pl. I, Fig. 5, fixed in Helly's fluid, which contains mercuric chloride) it is clear that the main mass of cytoplasm is organized into an aster which is radial to the center of the main mass of cytoplasm. As we saw above, this is the point radial to which concentration gradients must be organized.

Figures 6–11 of Plate I are cells of simple, single-layered epithelia. While in all simple epithelia there is a tendency for the filamentous mitochondria that are along the direct path of flow through the cell to be parallel to the path, the accuracy of the parallelism varies with the cell type. The mitochondria are straighter and more nearly parallel in those cells through which the amount of diffusion is greater. Thus the orientation is least marked in the glandular epithelial cells (Pl. I, Figs. 6, 7, 8), where surely there is slow diffusion; it is considerably more marked in the intestinal epithelium covering the villus (Pl. I, Fig. 11), where absorption processes must involve diffusion of a considerable volume of fluid through the epithelial cell; and the parallel orientation is most marked in the cell of the distal segment of the amphibian uriniferous tubule (Pl. I, Fig. 10), which is the part of the tubule through which there is the greatest water transfer (Walker and Hudson, 1937).

Thus, in cells where the cytoplasm consists of mitochondria suspended in hyaloplasm, with no visible complicating cell inclusions or cell organization, the orientation of mitochondria appears to be closely correlated with both the course and the amount of diffusion through the cell. From our consideration of asters, below, we shall see reasons for believing that these mitochondrial orientations are a consequence of the diffusion.

DISCUSSION

A. ORIENTATION OF MITOCHONDRIA

In the differentiated tissue cells we have described above we can only note the correlation between mitochondrial orientations and the course of diffusion. To discover the cause of this orientation it is necessary to examine its development. This has not been done with these tissue cells, but we may understand it from studies that have been made of aster formation in dividing eggs. The reorientations of granules in such eggs are quite comparable with the orientations of mitochondria (which, indeed, some of the granules are [Hibbard, 1922]); and in dividing spermatocytes, which contain only the filamentous mitochondria, these become oriented radially exactly as do the granules of the egg (Terni, 1914; Bowen, 1920; Pollister, 1930). Since it is of easy access, the cytoplasm of marine eggs has been intensively studied. It is densely packed with granules, among which extends the microscopically homogeneous hyaloplasm. The granules may be thrown to one side of the cell by centrifuging, and this reveals the hyaloplasm. As shown especially by the centrifuging experiments of Lillie (1908) and of Conklin (1917), this hyaloplasm, and not the visible granules, is responsible for many of the fundamental and distinctive physical characteristics of the protoplasm. It is responsible for the shape of the cell; it holds the nucleus in position. Within the hyaloplasm—even when it has been completely freed of granules—the asters and spindle of the mitotic apparatus develop and function. The hyaloplasm has a structure or framework which is elastic; and this elasticity is a property which restores cell shape and returns cell constituents to their original positions after their displacement by centrifuging. Orientations within the hyaloplasm (Conklin's spongioplasm) largely determine cell polarity, symmetry, localization of inclusions, etc. The visible inclusions in cells, such as granules and mitochondria, must be thought of as largely suspended in this hyaloplasm; and their movements are probably, for the most part, merely reflections of activity in the hyaloplasm. The development of an aster, as seen in the normal living eggs, is a good example of this last (Chambers, 1924). It forms in a region that contains densely packed granules. The aster becomes visible in this granular cytoplasm when its rays suddenly develop in the form of clear areas that are entirely free of granules and are radial to a point, the center of the aster. No structure is visible within the rays in the living cell. As one watches the egg, suddenly it is apparent that the granules have become rearranged, so that, whereas before they were densely and uniformly packed, now they are completely absent from certain areas, the astral rays.

From microdissection and centrifuging experiments we know something of the physical nature of the aster. As a whole, the aster is a gel; and it may be pushed about as a single mass by the micrurgist's glass needle. If two asters and a spindle are present, all three together form a highly viscous or gelated region. The centrifuge experiments record this highly viscous state of the aster by showing that it is more difficult to move granules through an egg that contains asters (Fry and Parks, 1934); the viscosity of the egg waxes with the appearance of an aster, and wanes with its disappearance. The astral rays are lines of flow within this gel, for Chambers has shown that fat droplets pushed by the needle into the ray, flow along it toward the astral center.

To return to tissue cells, the aster of the leucocyte must be physically much like the aster of a dividing cell.[1] It appears the same in fixed preparations; and, as we have seen above, radial flow, like the channels called "astral rays" in the dividing eggs, is to be expected from the concentration gradients in the centrally polarized leucocyte; furthermore, the nucleus of the leucocyte is concavo-convex instead of spherical—a fact best explained by assuming that it is molded upon the spherical gelated mass of the aster. We may then conclude that the aster of a leucocyte has arisen by the same sort of organization of the hyaloplasm as occurred in the egg or spermatocyte and that the mitochondria have a radial arrangement because they have been pushed out from the developing rays or channels of flow, just as the granules were moved about in the developing aster of the egg.

Thus the phenomena of aster formation show that, though involving submicroscopic elements in the hyaloplasm, the development of a channel of flow, an astral ray, is carried on with sufficient force to exclude from itself such formed structures of microscopic dimensions as egg granules and mitochondria. Indeed, in the orientation of the latter it seems an especially powerful force, for the mitochondria in the astral region are straightened out parallel with the line of flow (Pl. I, Fig. 3)—a considerable restriction of their normal tendency toward being sinuous bodies. Not only the arrangement but also the shapes of the mitochondria reflect the fact that the hyaloplasm of an aster in a dividing cell, or in a leucocyte, is organized into radial channels of flow in a viscous region.

It is the existence of the channels, or astral rays, that explains the mitochondrial orientation, instead of the reverse. One might, at first, think that the flow occurs in channels because the egg granules or mitochondria cause it to detour, as it were, and that then the more direct channels, involving less detour, would become predominant as the rays. If the channels were thus caused by the presence of the mitochondria, where there are no mitochondria or granules, we should expect no rays but only a sort of unlocalized general diffusion through the whole region of the aster. But, instead, examination of fixed preparations shows there are well-developed perfectly symmetrical asters in the granule-free areas of a centrifuged egg or in the mitochondria-free regions of spermatocytes, where the precocious nebenkern formation has concentrated all mitochondria in another part of the cell (Wilson and Pollister, 1937). In short, the fact that flow occurs in definitely organized channels must be ascribed to some property of the hyaloplasm itself; it is characteristic of the hyaloplasm to respond to diffusion by the organization of definite channels for flow, which are seen as astral rays in dividing cells and leucocytes.

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[1] There is, however, one important cytological difference: in the dividing cell the aster is formed under the influence of the centriole, while the leucocyte aster is a pure diffusion aster, probably not directly influenced by the centrioles.

From this analysis of conditions in the aster it is easy to explain the orientations in the epithelial cell. Here, also, the mitochondria are parallel with the course of flow; and this orientation unquestionably suggests that in the hyaloplasm of the epithelial cell the flow likewise takes place through definite channels, which are functionally strictly comparable with the astral rays. I propose to appropriate the *term* "canaliculi" for these assumed channels in epithelial cells.

The inner-ray material of the aster is, as we have seen, in a highly viscous or gel state. This state of asters and spindle makes nearly the whole dividing cell appear like a highly viscous jelly. It appears from the microdissections of Chambers and Renyi (1925) that the cytoplasm of epithelial cells is likewise, in most cases, a gel. Indeed, since this is known to be true also of nerve cells (Renyi, 1931) and of muscle cells (Speidel, 1938), it seems a very general rule that the cytoplasm of tissue cells is in a gel state.

Let us look at the pictures which, from cytological observations alone, we have thus far painted of the leucocyte and epithelial cells. The cytoplasm consists of hyaloplasm in a gel state and containing suspended visible formed bodies—mitochondria, Golgi apparatus, and centrioles. The mitochondrial orientations especially show us that the hyaloplasm, though apparently unorganized, actually has definite canaliculi, which are the direct channels for diffusion, for they are on a direct line between blood capillary and lumen end of the epithelial cell and on a direct line from cell periphery to center of the spherical leucocyte. Further details of the picture may be best filled in after consideration of the possible molecular basis of the hyaloplasmic structure.

B. STRUCTURE-PROTEINS

Bensley (Bensley and Hoerr, 1934, and Bensley, 1938) has recently given particular attention to the nature of the material that is the basis of cytoplasmic structure, that is, of the hyaloplasm; and he believes it to be due to a special protein fraction, for which I prefer his general term "structure-protein" to the more specific ones he also has suggested.

He extracts from liver cells a protein, which he calls "plasmosin." This very readily forms gels, and upon dilution it is precipitated out as a fibrous mass. From study of fresh tissue and of thin sections of frozen-dried material Bensley concludes that it is "beyond doubt that the plasmosin belongs to the apparently structureless ground-substance [i.e., hyaloplasm, A.W.P.] of the cell, not to membranes, mitochondria, or nuclear chromatin." And Bensley further suggests that since the units of plasmosin confer a certain degree of viscosity on the solution and since they readily collect into long fibers, it seems probable that they are of linear shape.

Very recently these structure-proteins of tissue cells have been briefly reported by

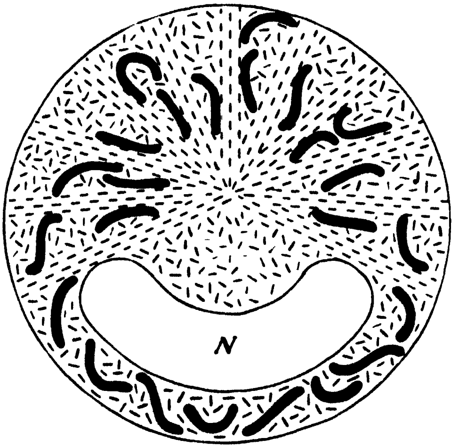
Banga and Szent-Györgyi (1940), who find, as did Bensley, that weak saline extraction removes only a part of the cell protein. The remainder they speak of as "structure-protein." This protein is a definite fraction analogous to myosin, that is, it is composed of rod-shaped molecules, as Bensley believed his plasmosin to be. Upon dilution it forms threadlike precipitates, as do plasmosin and also myosin (Weber, 1934). It is, furthermore, like myosin in the X-ray diffraction pattern and in that, in solution, it gives double refraction of flow.

It thus appears that tissue cells contain a considerable amount of structure-protein, of rod molecules like those of myosin or keratin. As Bensley has shown, this is the basis of structure of the hyaloplasm—or, as Banga and Szent-Györgyi say, it is "the solid edifice of the cell."

Furthermore, Mirsky (1936) has extracted a similar fraction as 12 per cent of the total protein of the unfertilized *Arbacia* egg. "In solution . . . . the protein particles appear to be exceedingly elongated, for the solution possesses high viscosity (a 1.4 per cent solution is 9.6 times as viscous as water), and the relative viscosity decreases when pressure is applied. Furthermore the solution shows double refraction of flow." Mirsky has shown that this protein is intimately involved with the establishment of increased strength and elasticity of the egg cytoplasm that follow fertilization. "The explanation of this increase in strength and elasticity is that in coagulation the elongated protein particles unite to form a fibrous network. This, an early step in the development of the egg, may be regarded as a skeleton framework within which differentiation proceeds."

The structure-protein is like myosin. There is considerable information about the behavior of rodlike molecules in general and about myosin in particular (see, e.g., Edsall, 1938; Mehl, 1938). When in the isotropic unoriented state, i.e., with the principal axes of the molecules free to orient at random, solutions of rodlike proteins form gels, even when very dilute (0.3 per cent in the case of myosin [von Muralt and Edsall, 1930]). These gels, called "thixotropic," have the property of responding to stress by a reorientation of their molecules parallel with the stress lines. Such orientation may be caused by mechanical stress, by stress due to flow, by fórces in an electrical field, etc. The larger the molecules the more readily will they orient. In dilute myosin gels the orientation manifests itself in two ways: first, the orientation destroys the isotropic gel state so that the myosin solution will flow; and, second, the oriented molecules doubly refract light between crossed Nicol prisms. Since this latter is readily detected by examining liquid under flow in a polarizer-analyzer apparatus, it is said to be a special property for identification of a thixotropic gel that it shows double refraction of flow. Refraction of flow at relatively low flow stress is also, of course, a practical test for the presence of large rodlike molecules. Solutions of myosin very readily show birefringence due to flow (von Muralt and Edsall, 1930). It is generally assumed that any birefringence in living cells is due to the presence of rodlike elements in a parallel arrangement (see Schmidt, 1937; Seifriz, 1936; Picken, 1940). Where protein with long rod-molecules is known to be present in considerable amount, one may reasonably assume that the birefringence is due to their parallel orientation. Thus the birefringence in parts of a striated muscle fiber is generally ascribed to some symmetrical arrangement of the myosin molecules.

Let us once more get back to tissue cells and dividing eggs. From the work of Bensley, of Banga and Szent-Györgyi, and of Mirsky it is evident that hyaloplasm contains a considerable amount of structure-protein, of long rod-molecules far in excess of that necessary to form a gel. Since both the leucocyte aster and the hyaloplasm of epithelial cells seem to be a gel (see p. 271, above), it may be tentatively assumed that this gel is due to the presence of the structure-protein and that it is of the thixotopic type,[2] with the rod-molecules oriented at random. The astral rays and epithelial canaliculi, however, are lines of flow, and this flow in a thixotropic gel should cause the rod-molecules of structure-protein to orient parallel to it. Possible molecular orientations in these two cell types are, then, as indicated in Text Figures 3 and 4. This orientation of long molecules parallel with the line of flow we may believe is somehow involved with the exclusion of the mitochondria from the rays and canaliculi.



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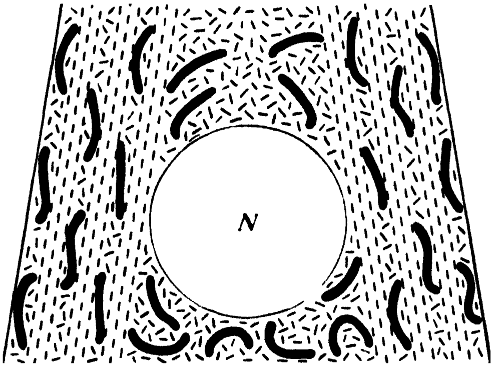

FIG. 3 —Leucocyte cell type. Suggested orientation of mitochondria and rodlike molecules of structure-protein. In the diffusion channels, the astral rays, molecules are oriented by flow and are parallel with the course of flow It is assumed mitochondria do not enter regions where molecules are thus oriented, but are confined to other parts of the cell where molecules are free to orient at random. *N*, nucleus.

FIG. 4.—Epithelial cell type. Suggested mitochondrial and molecular orientations. Positions of mitochondria, parallel with course of diffusion, suggests that there are definite channels of flow, canaliculi, in which molecules are oriented parallel with the course of flow—as in rays of leucocyte.

The presence of these lines of oriented molecules should be detectable by the birefringence effect; and Schmidt (1936*a*, 1936*b*) has found birefringence in asters of dividing eggs. Schmidt (1937) also cites some evidence of a birefringence in the cytoplasm of epithelial cells, as would be expected if the canaliculi consist of oriented molecules.

The view that the astral rays are lines of oriented molecules of structure-protein explains one apparent discrepancy between the observations on living cells and those on fixed preparations. In material fixed by strong protein coagulants the rays are more tenacious of the stain than are the interray regions. If the rays are, as has hitherto seemed most likely, watery channels, this heavier staining is quite unexpected; but it is not surprising if the rays are lines of oriented molecules. Coagulation might then readily cause the molecules to form a stainable, fiber-like artifact.

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[2] This assumption of a uniformly unoriented gel outside the rays and canaliculi is, of course, gratuitous. The stresses set up by distortion of a cell fitted into an epithelium must lead to special molecular orientations; and even the almost completely unknown effect of cellular surfaces on orientation may be very significant (see Hardy, 1928, 1932). Indeed, if one considers all these possible orienting forces, it would seem there could be no large intracellular protein molecule completely free to orient itself at random. In the cells we are here concerned with, however, the flow stresses seem to be very significant and to be those most likely responsible for the mitochondrial orientations, just as mechanical stresses surely are in developing muscle fibers (see below, p. 276); and so it seems a justifiable, and possibly more fruitful, simplification to consider the molecular orientations as if they were caused by these forces alone.

Since the canaliculi of the epithelial cell appear to be of the same nature as the rays, they also should stain after proper coagulation. They apparently do so, giving the expected picture of stained fiber-like bodies parallel with the line of flow through the cells. This is figured in every textbook of histology, where it is called "basal striation."

C. ORIENTATIONS IN OTHER TISSUES

In the striated muscle fiber it seems certain that the long myosin molecules are, in the mature fiber, arranged in a pattern that is the basis of the visible cross-striations—probably a much more elaborate arrangement than the simple parallel orientation in response to flow that has just been invoked to explain mitochondrial orientations in leucocytes and epithelial cells. As we should expect, the mitochondrial orientations reflect the molecular organization that is developing during the histogenesis of striated Muscle (Lewis and Lewis, 1917, and Friedheim, 1931, on skeletal muscle in tissue culture; Speidel, 1938, on regenerating skeletal muscle *in vivo;* Weed, 1936, on skeletal muscle in fixed preparations; and Goss, 1932, 1933, on cardiac muscle in tissue culture). When a muscle fiber is first capable of contraction, the mitochondria show no particular orientation; there are no myofibrillae or visible cross-striation, and no anisotropy. Molecular orientation is presumably then very slightly developed. As the differentiation proceeds, mitochondria first become parallel with the long axis of the cell. Then, as the cross-striations become visible, the mitochondria are moved about and fitted into this orientation pattern. From his careful observations of living cardiac muscle fibers Goss (1933) says: "We are of the opinion that the mitochondria are imbedded in a contractile matrix or sarcoplasm, and that some mechanical factor is responsible for their rearrangement rather than any direct influence they may have on the contractile power of the cell." It seems likely that, as was assumed for leucocytes, epithelia, and dividing cells, the mechanical factor operating in mitochondrial orientation in the developing muscle fiber is the organization of the myosin molecules into the fibrillar pattern, which must be occurring at this time. From the mitochondrial orientations we may infer that differentiation of the muscle fiber first causes the myosin molecules to assume a fairly simple orientation parallel with the long axis of the fiber. This stage seems to be that observed by the Lewises in muscle buds as a "fine striation which has in general a longitudinal direction," between which the mitochondria are arranged longitudinally. Friedheim has noted birefringence indicative of molecular orientation at this same stage. If the suggestions of Bernal (1938) as to the molecular orientation in muscle are correct, this first orientation may be like that of smooth muscle. Later the myosin molecules assume the much more intricate arrangement that causes the appearance of the alternate isotropic and anisotropic regions characteristic of the fully differentiated cross-striated fiber. In his studies of development of skeletal muscle Friedheim found different physiological properties associated with these two degrees of differentiation, only the second being capable of developing the tetanus characteristic of fully differentiated skeletal muscle. Speidel (1938) has observed that the viscosity is much greater after the cross-striation has appeared in regenerating skeletal muscle. From the experiments of

Weber (1934) with myosin fibers and from analogy with the effect of stretching on cellophane, which is unoriented regeneration cellulose (Seifriz, 1936), it may be concluded that the more nearly parallel molecular orientations in developing muscle confer greater tensile strength on the fiber; and this, in turn, must increase the force of the contraction—and perhaps also its extent. The Lewises (1917), in skeletal muscle, and more recently Goss (1933), in cardiac muscle, have noted that in tissue-cultured muscle the striations first appear in those cells or parts of cells that are subject to mechanical stress due to shape of the fiber and the consequent manner of its attachment to the edges of the clot in which the culture is growing. Goss observed that in the long type of fiber the striations appear nearly simultaneously throughout the whole fiber, with attachment points at either end; while in the irregular fiber, with several attachments, the differentiation occurs first in the areas of greatest stress and later spreads from these centers throughout the whole sarcoplasm. Rodlike molecules like those of myosin have the property of orienting parallel with mechanical stresses, and so what Goss has seen is to be expected. Since the development of cross-striated differentiation leads to increased viscosity and tensile strength and almost certainly to an accompanying increase in force of contraction, in the irregular cell the differentiated areas will serve the more readily to transmit the stresses to the undifferentiated regions and will subject these, in turn, approximately to the stresses that cause orientation of myosin molecules to cross-striated myofibrillae. This will thus involve, finally, the entire fiber.

Observations of the behavior of mitochondria in the differentiation of the neuroblast show that the mitochondria are forced into positions parallel to the neurofibrillae (see especially Hoven, 1910, Figs. 9, 10, 11, 14)—a case that appears to be strictly comparable with muscle histogenesis.

The myoblast may be easily pictured as a system whose structure, with function, must progressively differentiate in the direction of its final stage, the cross-striated muscle fiber. Its anchored ends exert stresses on the cell; each contraction is a momentary increase of the stress, which must certainly cause molecular reorientation parallel with the stress. A residue of each orientation must persist, causing the next contraction to be more powerful; and this increased stress, in turn, will cause more orientation at the next contraction. Eventually we can detect these orientations—first by their effect on the mitochondria, and finally also by the appearance of cross-striation. But the molecular orientation may be assumed to have been thus progressing ever since the first muscle twitch, which may, indeed, have occurred long before the contraction was of sufficient magnitude to be visible.

Thus viewed, the differentiation of cardiac muscle seems a suggestive simple model of histogenesis and tissue determination. If it be assumed, first, that undetermined mesenchyme cells all contain protein that is potentially myosin and, second, that the amount of molecular orientation caused by a few submicroscopic contractions is sufficient to set a mesenchyme cell, if under mechanical stress, on its way to becoming a muscle fiber, then we have a mechanism for determination of this specific tissue.

SUMMARY

The observations show that there are many cells that contain neither stored material nor visible fibrillar differentiations but which, nevertheless, show the mitochondria oriented in a very definite manner. Analysis of this orientation in epithelial and leucocyte types of cells shows that the threadlike mitochondria are parallel to the course of

diffusion in the cell—on a line between capillary and lumen in the former—and radial to the approximate center of the main mass of cytoplasm, which is organized into an aster, in the latter.

It has been shown that the process of formation of astral rays pushes out formed bodies, including mitochondria, from the rays. Furthermore, astral rays are channels of flow. It is concluded, therefore, that the orientation of mitochondria in leucocytes and epithelia is similarly due to their being excluded from special channels of flow—the demonstrated astral rays in the leucocyte, and assumed channels, canaliculi, between basal and lumen ends of epithelial cells.

These orientations are held to be due to the fact that the mitochondria are excluded from regions of rays and canaliculi by the assumption of parallel orientation by long protein molecules—the structure-proteins—of the hyaloplasm. This view finds support in the literature from (*a*) observations of differentiating muscoe and nerve cells, where the mitochondria are similarly forced into positions parallel to the lines of molecules that are becoming oriented into myofibrillae and neurofibrillae; (*b*) the fact that astral rays stain heavily, though known to be channels of flow; and (*c*) evidence of birefringence in asters and in epithelial cells.

LITERATURE CITED

BANGA, I., and SZENT-GYORGYI, A. 1940. Structure-proteins. Science, 92:514–15.

BENSLEY, R. R. 1938. Plasmosin. The gel- and fiber-forming constituent of the protoplasm of the hepatic cell. Anat. Rec., 72:351–69.

BENSLEY, R. R., and HOERR, N. L. 1934. The chemical basis of the organization of the cell. Anat. Rec., 60:251–66.

BERNAL, J. D. 1938. A speculation on muscle. Perspectives in biochemistry. Cambridge University Press.

BOWEN, R. H. 1920. History of the cytoplasmic components of the sperm in Hemiptera. Biol. Bull., 39:316–62.

CHAMBERS, R. 1924. The physical structure of protoplasm. General cytology. Chicago: University of Chicago Press.

CHAMBERS, R., and RENYI, G. S. 1925. The physical relationships of the cells in epithelia. Amer. Jour. Anat., 35:385–402.

CONKLIN, E. G. 1917. Effects of centrifugal force on the structure and development of the eggs of *Crepidula*. Jour. Exper. Zoöl., 22:311–419.

EDSALL, J. T. 1938. Anisotropy. Chap. ix, sec. xii, in C. L. A. Schmidt (ed.), Chemistry of amino acids and proteins. Springfield, Ill.: Thomas.

FRIEDHEIM, E. A. H. 1931. Morphologische und funktionelle Untersuchungen an isolierten, in vitro gezüchteten, Skelettmuskelfasern. Arch. f. exper. Zellforsch., 11:385–96.

FRY, H. J., and PARKS, M. E. 1934. Mitotic changes and viscosity changes in eggs of *Arbacia*, *Cumingia*, and *Nereis*. Protoplasma, 21:473–99.

GOSS, C. M. 1932. Formations of cross-striations in cultures of embryonic heart muscle. Arch. f. exper. Zellforsch., 12:233–48.

———. 1933. Further observations on the differentiation of cardiac muscle in tissue cultures. *Ibid.* 14:175–201.

HARDY, W. B. 1928. Molecular orientations in living matter. Jour. Gen. Physiol., 8:641–43.

———. 1932. Adsorption. A study of availability and accessibility. Proc. Roy. Soc., London, A., 138: 259–83.

HIBBARD, H. 1922. Cytoplasmic inclusions in the egg of *Echinarachinus*. Jour. Morph., 36:467–94.

HOVEN, H. 1910. Sur l'histogénèse du systeme nerveux periphérique chez le poulet. Arch. de biol., 25: 426–92.

LEWIS, W. H., and LEWIS, M. R. 1917. Behavior of cross-striated muscle in tissue cultures. Amer. Jour. Anat., 22:169–93.

LILLIE, F. R. 1908. Polarity and bilaterality of the annelid egg. Biol. Bull., 16:54–79.

Mehl, J. W. 1938. Double refraction of flow of protein solutions. Cold Spring Harbor symposia on quantitative biology, **6**:218-27.

Mirsky, A. E. 1936. Protein coagulation as a result of fertilization. Science, **84**:333–34.

Muralt, A. L. von, and **Edsall, J. T.** 1930. The anisotropy of myosin and double refraction of flow. Jour. Biol. Chem., **89**:351–86.

Picken, L. E. R. 1940. The fine structure of biological systems. Biol. Rev., **15**:133–68.

Pollister, A. W. 1930. Cytoplasmic phenomena in the spermatogenesis of *Gerris*. Jour. Morph., **49**: 455–507.

———. 1932. The cytology of the liver of *Amphiuma*. Amer. Jour Anat., **50**:179–98.

———. 1933*a*. Studies on the cytology of Amphibia. Biol. Bull., **55**:358 (abstract).

———. 1933*b*. Centrioles of amphibian tissue cells. *Ibid.*, **65**:529–45.

———. 1939. Structure of the Golgi apparatus in the tissues of Amphibia. Quart. Jour. Mic. Sci., **81**: 235–71.

Renyi, G. S. 1931. The physical properties of the nerve cells of the frog. Jour. Comp. Neurol., **53**:497–509.

Schmidt, W. J. 1936*a*. Kernspindel und Chromosomen in lebenden sich furschenden Ei von Psammechinus miliaris (Müll.). Ber. oberhess. Gesellsch. Nat.- u. Heilk., **17**:140–50.

———. 1936*b*. Doppelbrechung von Chromosomen und Kernspindel in der lebenden Zelle. Naturwiss., **24**:463–70.

———. 1937. Die Doppelbrechung von Karyoplasma, Zytoplasma, und Metaplasma. Protoplasma Monographien, No. 11. Berlin: Borntraeger.

Seifriz, W. 1936. Protoplasm New York: McGraw-Hill.

Speidel, C. C. 1938. Growth, injury, and repair of striated muscle. Amer. Jour. Anat., **62**:179–235.

Terni, T. 1914. Condriosomi, idiozoma, e formazioni periidiozomiche nella spermatogenesi degli anfibii. Arch. f. Zellforsch., **12**:1-96.

Walker, A. M., and **Hudson, J.** 1937. Role of the tubule in urea secretion in the amphibian kidney. Amer. Jour Physiol., **118**:153–70.

Weber, H. H. 1934. Die Muskeleiweisskörper und der Feinbau des Skelettmuskels. Ergebn. d. Physiol., **36**:109–50.

Weed, I. G. 1936. Cytological studies of developing muscle. Zeitschr. f. Zellforsch., **25**:516–40.

Wilson, E. B, and **Pollister, A. W.** 1937. Observations on sperm formation in the centrurid scorpions. Jour. Morph., **60**:407–43.

PLATE I

Camera lucida drawings of cells of urodelous Amphibia. In Figs. 6–11 the free (distal or lumen) end of each cell is toward the top of the page; and the lower border of each cell is the basal or proximal end, that adjacent to the blood capillaries.

FIG. 1.—Hepatic epithelium, *Triturus*. In autumn, when cell is filled with stored material in the form of droplets of fat, blackened with osmic acid; and clear spaces, which were formerly occupied by glycogen. Mitochondria restricted to hyaloplasm between these masses of stored material. Kolatchev method. ×2,100.

FIG. 2.—Fibroblast, *Amblystoma opacum* larva. Kull method. ×2,100.

FIG. 3.—Leucocyte, perihepatic zone, *Amphiuma tridactylum*. Small sphere and adjacent black dot are centrosome and centrioles. Kull. ×2,100.

FIG. 4.—Cartilage cell, *Amblystoma opacum* larva. Two small black dots are centrioles. Nucleus not in section. Benda. ×2,100.

FIG. 5.—Leucocyte, like Fig. 3 but fixed in Helly's fluid and stained with iron haematoxylin. ×2,100.

FIG. 6.—Goblet cell, duodenum, *A. opacum* larva. Kull. ×1,200.

FIG. 7.—Pancreas, gland cell, *A. opacum* larva. At upper, lumen, end are prezymogen granules. Kull. ×1,200.

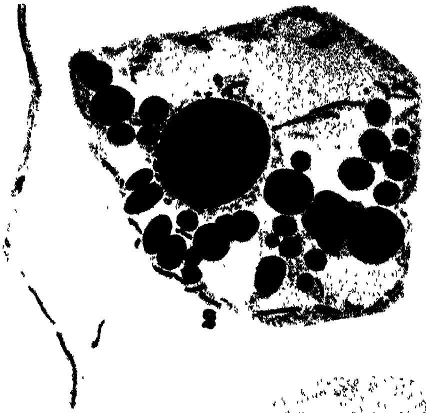
FIG. 8.—Gastric gland cells, *A. opacum* larva. Kull. ×1,200.

FIG. 9.—Epithelial cell, proximal segment of kidney tubule, *Triturus*. Kull. ×2,100.

FIG. 10.—Epithelial cell, distal segment of kidney tubule. *Necturus*. Kull. ×1,200.

FIG. 11.—Epithelial cell, duodenal villus, *Necturus*. Small granules are not mitochondria. Kull. ×2,100.

PLATE I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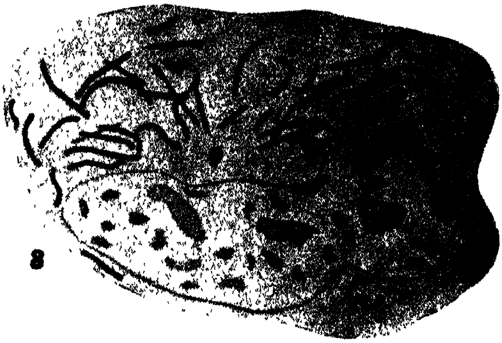
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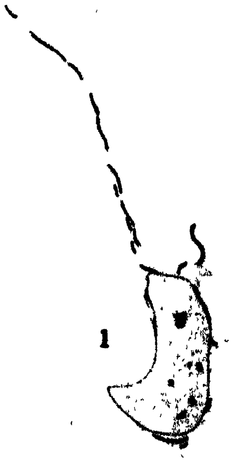
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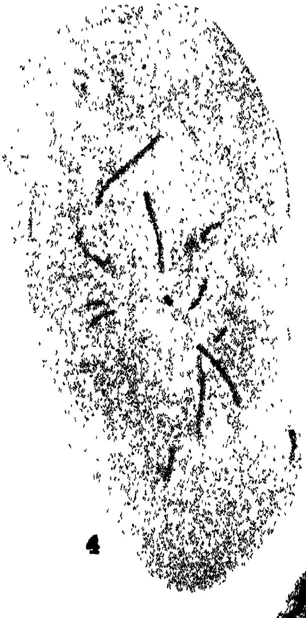
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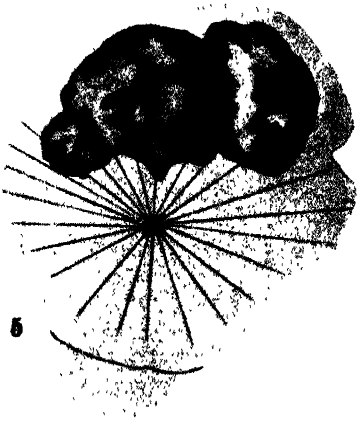
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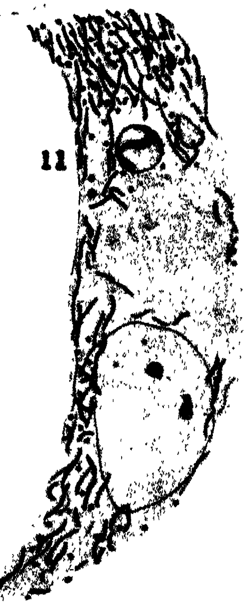
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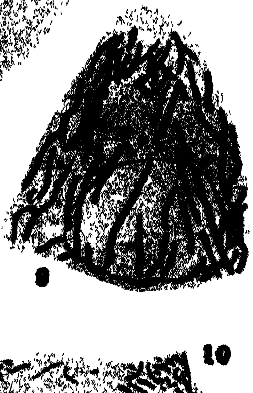
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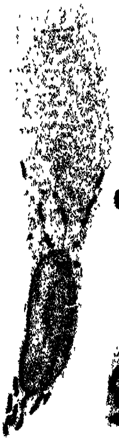
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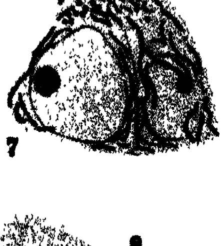
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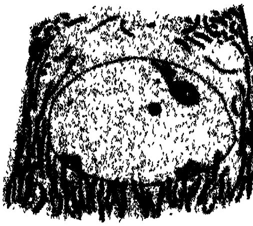
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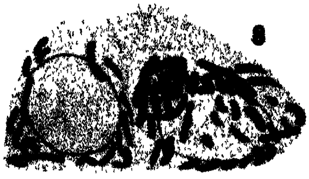
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THE IRRITABILITY OF FROG MUSCLE IN RELATION TO THE SODIUM ION[1]

(Two figures)

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AMONG physiologists generally, it is commonly believed that the irritability of striated muscle is primarily dependent on the presence of sodium ion. This belief is based on a remarkable paper published thirty-nine years ago by Overton and never, to the best of our knowledge, seriously challenged. Overton's work is so impressive that it is easy to understand why it has had such a lasting influence. Standard books in the field (e.g., Höber, 1926; Lillie, 1932) provide extensive summaries of Overton's experiments, and they accept his conclusions without question.

What Overton did was essentially this: Frog muscles were suspended in approximately isotonic solutions of sucrose and other sugars. In such solutions they lost their power to respond to direct stimulation with an induction coil. Small muscles, such as the sartorius, became nonirritable in a relatively short time (in as little as an hour or less); however, the gastrocnemius required somewhat longer immersion before its irritability was lost. Following loss of irritability, when the muscles were returned from sugar solutions to solutions of sodium chloride or other sodium salts, direct excitability was regained. More than any other cation, sodium was effective in restoring the irritability lost in sugar solutions; lithium was also effective; but calcium was relatively without effect. Indeed, in his 1902 paper Overton stated that irritability could not be restored by calcium, but later (1904) he retracted this statement and admitted that irritability lost in sugar solutions could, to some extent, be restored by calcium if the proper concentration of calcium was correctly hit ("passend getroffen").

In his first attempt to explain the action of sugar Overton decided that sugar acted by replacing with a nonconducting solution the electrolyte medium which normally surrounds the muscle fiber, thus rendering electric stimulation ineffective because of failure of the stimulating current to reach the tissue. He was so sure of this interpretation that he called in his teacher (von Frey) to witness a proof of the hypothesis. A nerve-muscle preparation was rendered nonirritable by immersion in a sugar solution; then the nerve alone was removed from the sugar solution, immersed in a sodium chloride solution, and stimulated electrically. Overton apparently expected the nerve to regain its irritability as soon as the sodium chloride was able to conduct the electric current to the nerve fibers; and he expected, further, that if the nerve fibers were aroused the muscle would, of necessity, contract. The fact that the muscle did not contract indicated to Overton that his (original) hypothesis was incorrect, and he concluded that the sugar must render the individual muscle fibers nonirritable. Further evidence in support of this view came from the observation that the nerve to the gastrocnemius muscle did not lose its irritability even after long immersion in sugar solution. It is, of course, obvious that loss of indirect excitability of a nerve-muscle preparation may be due to loss of irritability of the nerve,

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the muscle, or the myoneural junction; but, strangely enough, throughout his 1902 paper Overton never considers the possibility that loss of indirect excitability may depend, in part at least, on the myoneural junction. Later, in 1904, Overton does discuss the myoneural junction at considerable length, but by this time he had completely abandoned his original hypothesis.

Actually there are two separate questions to be answered. In the first place, is the direct excitability of the muscle cells themselves primarily dependent on sodium ion? And, secondly, is the indirect excitability of muscle—that is to say, its response to stimulation through its nerve—primarily dependent on sodium ion?

To the first of these questions Overton's answer was simple. He held that in sugar solutions muscle fibers lose their irritability because sodium ion is removed from the tissue spaces which constitute the immediate environment of the fibers, and that replacement of sodium results in a restoration of irritability.

Concerning the indirect excitability of muscle, there can now be no question. It is primarily dependent on calcium ion rather than on sodium ion. Indeed, in sodium chloride solutions indirect excitability is lost and can only be recovered when calcium ion is added. This is indicated by the early work of Locke (1894) and Cushing (1901). Overton was unaware of this early work when he published his 1902 paper, but he knew of it by 1904 and was able to confirm it. Since then it has received frequent additional confirmation (see Poliakoff, 1904; Joseph and Meltzer, 1911; Kleefeld, 1913; Taubmann and Hikiji, 1931; Lapicque and Lapicque, 1933; Taubmann, 1934; Feng, 1936; Chao, 1936, 1937; Cicardo, 1938). Moreover, in experiments of our own on sartorius and semitendinosus muscles we have obtained convincing proof that calcium ion must be present if indirect excitability is to be maintained. Muscles which have lost their indirect excitability following immersion in sugar solution for 20–30 minutes recover it only when returned to solutions containing calcium ion. In view of the general agreement concerning the need of calcium ion for indirect excitability it is scarcely necessary for us to submit a complete description of these experiments, and we shall confine our attention to the question of the relation of direct muscle excitability to the presence of sodium ion.

CORRECTNESS OF OVERTON'S BASIC EXPERIMENT

It is a simple matter to show that, if a frog muscle is immersed in an isotonic solution of sucrose, after a time it gives no obvious response when stimulated directly with an induction coil; it is also easy to show that, following transfer from the sugar solution to a solution of sodium chloride, the same electric shocks which were previously without obvious effect now produce well-marked contractions of the muscle. Observations of this sort have been made not only by Overton but also by various other authors, including Schwartz (1907), Höber (1907), and Chao (1937). They can readily be confirmed, and *from the outset we realized that Overton's basic experiment was undoubtedly correct in so far as the facts go.*

Although Overton reported his experiments in detail, his observations on the length of time necessary for loss of irritability are not always clear. Thus, in some instances he stated that a muscle was nonirritable at a certain time, without giving any assurance that this particular time was the exact moment or even the approximate moment at which irritability was lost. For sartorius muscles in sucrose solutions Overton (1902) notes the following times for loss of direct excitability: 45, 30, and 120 minutes. In one experiment with glucose solution a sartorius muscle lost its irritability in 40 minutes.

These are the only observations recorded in the 1902 paper for sartorius muscle. Loss of irritability occurred more rapidly in the muscles of the toes, especially the smaller toe muscles. Gastrocnemius muscles took longer times to lose their irritability. In the 1904 paper the times required for loss of irritability in sugar solutions were longer, with the exception of one case (Expt. 13) in which apparently only an approximate time was given. The actual data are 60, 300, 114, 258, 182, 140, 87, 102, and 147 minutes for sartorius muscle. Some of the longer times are certainly due to infrequency of observation, but in one case (Expt. 22) Overton states that the muscle was still irritable after 115 minutes' immersion. It is thus incorrect to say, as do Fenn (1931) and Victor (1933), that Overton showed loss of irritability of muscles in sugar solutions in 15 minutes.

As a matter of fact, the time required for a muscle immersed in sugar to lose its irritability is largely a function of the method of observation. When a muscle is first placed in a sugar solution, it undergoes violent twitching and contracture. After the twitching ceases, if the muscle is then tested by any sort of electric stimulus, its reponse is decidedly less than that which a similar shock would have produced if the muscle were in Ringer's solution or in a solution of sodium chloride. If the shocks are repeated at intervals of several minutes, before long there is only a localized response, and this gradually becomes less and less visible. It is often difficult to determine exactly when the last traces of response disappear. In our experiments we observed the response of the muscle with a low-power microscope, and under such conditions the times required for complete loss of response are typically higher than any recorded by Overton. The last traces of irritability can generally be observed only in a few fibers in the vicinity of the electrodes. Moreover, the region of the muscle around the anode has a higher threshold than the region around the cathode.

IRRITABILITY OF INDIVIDUAL FIBERS AFTER PROLONGED IMMERSION OF MUSCLES IN SUGAR SOLUTION

The fact that in sugar solution the response of a muscle may become extremely localized leads to the suspicion that there may be an element of truth in Overton's original hypothesis. The fibers close to the electrodes respond to an electric shock presumably not because they are more sensitive than other fibers of the muscle but because the electric current reaches them and does not reach more remote fibers. This is not the complete explanation of the effect of the sugar solution, for an additional factor is almost certainly the failure of muscle fibers themselves to conduct the response to stimulation from one region of the fiber to another.

If Overton's original hypothesis is in part correct, then after muscles have been immersed for long times in sugar solutions it should be possible to dissect from such muscles fibers still sensitive to electric stimulation. This is really a very simple experiment to perform. After muscles have been in sugar solutions for a long time, fibers are teased out from them with ordinary dissecting needles, and the irritability of these individual fibers can then be tested. Experiments of this sort are reported in Tables 2 and 3, which appear in a later section of this paper. The experiments of Table 3 are the more convincing. In 13 experiments of this table sartorius and adductor magnus muscles were immersed in isotonic solutions of sucrose for longer than an hour, and yet in every case fibers isolated from the muscles proved irritable. In 2 cases the individual fibers were irritable, even though the muscles from which they had been taken had spent over 4 hours in the sugar solution.

In Tables 2 and 3 the thresholds of the whole muscle in sugar solution are also given, and it is to be noted that irritability persists for long times. However, as previously stated, these determinations were made under a microscope, and the threshold voltages concern only the contraction of very few fibers close to the electrodes. It is thus apparent that when muscles are immersed in sugar solutions individual fibers retain their irritability for much longer times than Overton supposed.

The conclusion to be derived from such observations seems inescapable. In Overton's experiments failure of sartorius muscles to respond to stimulation after immersion in sugar solutions for an hour or two may well have been due largely to the fact that the applied electric current did not reach individual fibers remote from the electrodes.

IF OVERTON'S ORIGINAL HYPOTHESIS IS PARTLY CORRECT, THEN WHY DOES NOT ANY ELECTROLYTE ACT LIKE SODIUM CHLORIDE?

Clearly, the transfer of muscle from sugar solution to a solution of any electrolyte should provide a good conducting medium in and around the individual fibers of the muscle. Accordingly, if Overton's original hypothesis is correct, transfer from sugar solution to any electrolyte solution should quickly restore response to electric stimulation. Overton's experiments indicated that this was not the case. And yet these experiments do not constitute a disproof of his original hypothesis, according to which response fails to occur in a nonelectrolyte because of the failure of the stimulus to reach the tissue. The reason that sodium chloride is distinctive in restoring irritability may lie in the fact that sodium is relatively nontoxic. If a muscle, after having lost its irritability in sugar solution, is transferred to a solution of potassium chloride, one could scarcely expect the irritability to return, for potassium itself can rob a muscle of irritability. According to Höber (1926), this has been known since the time of Claude Bernard.

On the other hand, if a muscle is transferred from a sugar solution to a solution of calcium chloride, pronounced shortening occurs. The shortening might tend to prevent additional contraction on stimulation by an electric current. If, instead of transfer to isotonic calcium chloride solution, transfer is made from the sucrose solution to a solution of sucrose plus a small percentage of isotonic calcium chloride solution, then, if Overton's original hypothesis were correct, one might expect that under favorable conditions there would be at least a transitory return of irritability. This is exactly what does occur, as Overton himself found (see Overton, 1904, p. 222) and as we have confirmed. See also Kleefeld (1913).

Although calcium in proper concentration can cause a return of muscle irritability following immersion in sugar solution, this return is never as complete as that obtained in solutions of sodium salts. Later we shall cite experiments to show that, for the isolated single fiber, calcium is far more effective than sodium in restoring irritability following immersion in sugar solutions. Indeed, sodium is not effective at all. It is thus evident that the behavior of the single fiber is different from that of the whole muscle. The reason for this difference is not wholly clear. It is obvious, however, that the entire muscle is a complex system in which various secondary effects may be produced. We believe, therefore, that experiments on single fibers yield results of greater significance than those obtained on the whole muscle.

THE RELATION OF THE SODIUM ION TO RESPONSE TO MECHANICAL STIMULATION

In a sentence or two at the end of his 1902 paper Overton cites two arguments against his original hypothesis. Loss of irritability in sugar solutions, he states, cannot be due to increase of electrical resistance, for the muscle in sugar solution is also nonirritable to mechanical stimuli. And, second, irritability to electric stimuli is only very slightly lowered in mixtures of sugar and sodium chloride solutions, and not until the salt concentration falls below 0.12 per cent does a sharp drop in irritability occur.

The second of these arguments does not seem very important to us. The relation of a muscle to a stimulating electric current is a complicated one. With decrease in the conductivity of the solution in which the muscle and its fibers are bathed, there is a decrease in the total amount of current passing through the system; but, at the same time, with decrease in the conductivity of the fluid surrounding the fibers, a greater proportion of the current is shunted through the fibers and less of it passes between the fibers. Thus, as salt concentration is lowered, the first factor tends to decrease the effect of an electric current, whereas the second factor tends to increase it. With a system of two opposing factors such as these, it is hard to know just what is happening. Certainly it is quite conceivable that a sharp drop of irritability when the salt concentration falls below a certain point may be due to purely electrical factors.

On the other hand, Overton's first point is an important one, in spite of the fact that it is based only on two recorded experiments (Nos. 10 and 11). In these experiments Overton's primary concern was in another direction. Nevertheless, he did show that, following immersion in sugar solution, muscles failed to respond to the mechanical stimulation resulting from ligature with a silk thread. Moreover, on return from sugar solution to a solution containing sodium chloride, irritability to mechanical stimulation (by ligature) was recovered.

It can readily be shown that Overton was correct in stating that irritability to mechanical stimulation is lost in sugar solution and recovered in solutions of sodium chloride. Moreover, such irritability is not recovered in solutions of potassium or calcium chloride, for experiments of our own have demonstrated this fact.

A point which Overton did not recognize is that irritability to mechanical stimulation is lost much more rapidly than irritability to electrical stimulation. This is shown by a series of experiments in which muscles, on being placed in sucrose solution (0.234 molal), were stimulated at short intervals by being struck a sharp blow with the edge of an ordinary microscope slide. This type of mechanical stimulation is easier to apply than a ligature and apparently produces less injury. In Ringer's solution, when a sartorius muscle is struck while it is being observed under the microscope, it is easy to see that fibers in the neighborhood of the struck area show irregular and periodic twitching. There may also be some contracture localized in the immediate neighborhood of the blow. Table 1 shows the time necessary for loss of response to mechanical stimulation following immersion in isotonic sucrose solutions.

It is clear that loss of irritability to mechanical stimulation occurs very rapidly in sucrose solutions—far more rapidly than loss of irritability to electric stimulation. Perhaps this means little more than that mechanical stimulation is not as powerful as strong electrical stimulation, but to us such an interpretation seems somewhat doubtful.

Certainly, although Overton reported only 2 experiments with mechanical stimulation, his case for the necessity of sodium salts seems much stronger for this type of stimulation than it is for electrical stimulation. It would be interesting to discover if the individual fibers of a muscle immersed in sugar solution retained irritability to mechanical stimulation. Such an experiment is not easy to perform, since in our experience any mechanical stimulus strong enough to evoke a response of any sort, usually a localized contracture, also caused a visible injury. This injury appeared to be a localized, largely irreversible shortening, closely resembling the plug formed at the cut ends of a fiber in the presence of calcium (Heilbrunn, 1940).

It might be possible, therefore, to assume that mechanical stimulation produces slight local injuries and that these injuries in themselves are the direct cause of the response. Immersion in sugar, with consequent removal of calcium ion, should therefore prevent response to mechanical stimulation, for it is the calcium ion which is primarily responsible for the plug reaction. Against such an interpretation is the fact, readily observable, that a muscle which has lost its irritability to mechanical stimulation by immersion in sugar solution regains its irritability on being placed in a solution of pure sodium chloride. However, it is possible that when the muscle is placed in the sodium chloride solution, after the brief immersion in sucrose necessary for loss of mechanical irritability, some calcium is still available within the muscle and is released. This notion of calcium release by sodium solutions was evoked by earlier students of ion physiology to explain some of the puzzling phenomena they encountered (see, e.g., Martin, 1904, 1906). Recently, there has been some direct evidence to show that bound calcium in cells is actually set free by pure sodium chloride solutions (Mazia, 1933), just as in blood, calcium is set free from calcium proteinate by sodium ion (Loeb, 1924; Loeb and Nichols, 1927).

TABLE 1

MECHANICAL STIMULATION

A				B			
Time for Loss of Irritability in Sucrose* (Minutes)	Duration of Immersion in Sucrose (Minutes)	Recovery Medium	Time for Return of Irritability (Minutes)	Time for Loss of Irritability in Sucrose (Minutes)	Duration of Immersion in Sucrose (Minutes)	Recovery Medium	Time for Return of Irritability (Minutes)
sa. 7	20	NaCl	8	7	20	Na oxal.	7
st. 7	20	NaCl	7	17	20	Na oxal.	no recovery
st. 6	10	NaCl	4.5	6	10	Na cit.	16
il. 6.5	10	NaCl	5	6.5	10	Na cit.	no recovery
sa. 5	64	NaCl	8	5	64	Na cit.	no recovery
il. 7	62	NaCl	9	7	61	Na cit.	no recovery
sa. 10	12	NaCl	2.5	12	13	Na cit.	2.5
st. 12	13.5	NaCl	6.5	14	15	Na cit.	no recovery
sa. 29	29	$NaNO_3$	20	30	30	Na cit.	no recovery
sa. 19.5	20.5	$NaNO_3$	4	21.5	21.5	Na cit.	no recovery
st. 18	19	$NaNO_3$	3	20	20	Na cit.	no recovery
st. 17.5	17.5	$NaNO_3$	11	19	19.5	Na cit.	no recovery

* sa. = sartorius; st. = semitendinosus, il. = ileofibularis.

There is also a possible explanation in terms of electrical theory. If mechanical stimulation must cause injury to produce an effect, then the response may be essentially due to the electric currents which arise as a result of the injury. Such currents would cease to flow when the muscle is immersed in a nonconducting sugar solution but would be restored on return to a sodium chloride solution. This type of interpretation would obviously be an extension of Overton's original hypothesis. Injury currents in muscle have voltages of 50 millivolts or more, and such voltages may be enough to cause response, especially in cases in which mechanical impact causes disturbances in position of the fibers, so that several injured fibers might have their currents arranged in series.

Although it is possible that mechanical stimulation, to be effective, must involve localized injuries of individual muscle fibers, this is not a thesis we should like to uphold as constituting a complete explanation of the facts. There is another type of interpretation which is perhaps more important. In the first place, it should be recognized that sodium ion and mechanical shock both have similar effects on muscle. It has long been known that a muscle immersed in a pure solution of sodium chloride will respond by twitching. In such a response individual fibers of the muscle contract independently of each other to give the characteristic appearance of fibrillation well known to muscle physiologists. Following mechanical stimulation, the response is, to some extent, similar. Moreover, muscles which have lost irritability to mechanical stimulation by brief immersion in sugar solution, on being placed in sodium chloride solutions begin to show spontaneous twitchings at about the time they regain their irritability to mechanical stimulation. In such muscles placed in sodium chloride solutions following loss of irritability in sugar solutions, it can be assumed that the sodium ion acts synergistically with the mechanical stimulation. In other words, the twitchings which tend to be initiated by the sodium ion alone are enhanced by mechanical stimulation, and vice versa (compare Adrian and Gelfan, 1933). Now it has been shown that the response of an isolated muscle fiber to sodium ion depends on the presence of calcium ion in the surface or cortex of the fiber (Ashkenaz, 1941). It seems likely, therefore, that response to mechanical stimulation is also dependent on the presence of calcium ion in the cell cortex.

We have performed one experiment which favors this interpretation. If, as Overton believes, it is the sodium ion alone that is essential for the contraction of muscle, then it should be immaterial which sodium salt is added to restore irritability. However, if muscles which have lost their irritability to mechanical stimulation by brief immersion in sugar solutions are placed in isotonic solutions of various sodium salts (chloride, nitrate, oxalate, citrate), then the chloride and nitrate restore irritability to mechanical stimulation in a few minutes, whereas the citrate and oxalate, which tend to remove active calcium ion from solution, do not generally favor restoration of irritability. This is shown in Table 1, which represents experiments on matched pairs of muscles. The muscles in the A group were exposed first to isotonic sucrose solution and then to an isotonic solution of either sodium chloride or sodium nitrate. The corresponding muscles of the B group were transferred from the sugar solution to isotonic solutions of sodium oxalate or sodium citrate. In all 12 experiments, return to sodium chloride or sodium nitrate resulted in a restoration of irritability to mechanical stimulation. On the other hand, in 9 out of the 12 cases no restoration of irritability occurred when the muscles were transferred from the sugar solution to the oxalate or citrate solution.

These experiments on mechanical stimulation lead to no very definite conclusions as to the need for sodium ion in the response of the muscle fibers to mechanical shock. For one thing, we have not been able to exclude the possibility that mechanical stimulation may depend on injury. It is true that solutions of sodium chloride restore irritability to mechanical stimulation after this has been lost by immersion in sugar solutions. It is possible that the restorative effect of the sodium chloride solution may be due to a release of calcium ion to the intercellular spaces of the muscle. It is possible, also, that the sodium chloride may cause a release of calcium ion within the substance of the muscle fibers themselves. In support of such a possibility is the fact that the stimulating action of sodium ion on cells can generally be interpreted as being due to a release of calcium to the cell interior, as a result of the action of the sodium ion on bound calcium present in the cell cortex (see Heilbrunn, 1937, p. 378, 438; also Heilbrunn and Wilbur, 1937). If, following brief immersion of muscles in sugar solution, transfer to sodium chloride solution did cause a release of calcium ions to the interior of the muscle cells, then the sodium ion might be thought of as acting synergistically with mechanical stimulation; and, indeed, sodium ions and mechanical stimulation produce somewhat similar effects on muscle. The fact that sodium oxalate and sodium citrate typically do not restore irritability to mechanical stimulation is an indication that the calcium ion may be necessary—if not in the medium, at least in the fiber itself. Further study will eventually clarify the situation. For the present, one cannot state with any assurance what the role of the sodium ion may be in mechanical stimulation. As will be noted later, the results for electrical stimulation are much clearer.

THE RELATION OF THE SODIUM ION TO THE RESPONSE OF ISOLATED MUSCLE FIBERS TO ELECTRICAL STIMULATION

In an earlier section it was noted that, when frog muscles were immersed in isotonic sucrose solution for times sufficient to insure loss of visible response to stimulation by induction shock or condenser discharge, that, following such loss of irritability, the individual fibers, on being dissected from the muscle, usually responded to electrical stimulation even though still immersed in sucrose solution. Now the question arises as to the effect of sodium ion on the irritability of such isolated fibers. If Overton's major point is correct, then sodium ion—and sodium ion alone—should favor the response of these fibers to electrical stimulation. This is perhaps the crux of the whole problem. We shall present evidence to show: first, that the excitability of the isolated muscle-fiber preparation is favored by calcium ion alone; second, that sodium ion decreases the excitability; and, third, that the depressing effect of sodium on excitability can be neutralized by the addition of calcium ion.

First as to method. Immediately after a frog was pithed, the muscles desired for study were placed either in an isotonic sucrose solution (0.234 molal) or in Ringer's solution. The muscles used were sartorius and adductor magnus. The Ringer's solution consisted of the following: 0.65 gm. NaCl, 0.02 gm. KCl, 0.026 gm. $CaCl_2 \cdot 2H_2O$, distilled water to 100 cc. This was buffered by adding M/15 Na_2HPO_4 to pH 7.3–7.5. The concentration of the sodium chloride solution used was 0.73 per cent (approximately M/8). Such a solution is isosmotic with frog blood (Hill and Kupalov, 1930). Calcium chloride was used in 0.09 M solution. This is isosmotic with the sodium chloride solution, as determined by calculation from freezing-point data.

After a little practice isolated muscle fibers are easy to obtain, especially from the adductor magnus muscle. The individual fibers are teased away from their neighboring fibers by the use of ordinary dissecting needles. Then a convenient length of the fiber is cut and is ready for study. As a result of the method of preparation the fiber is cut at both ends and is therefore not wholly normal. Indeed, from the moment of preparation it begins to die from both cut ends. Certainly a superior preparation from the standpoint of normality is that of Ramsey and Street (1938). However, the Ramsey and Street preparation takes far longer to prepare and is therefore not so convenient for extended series of experiments. If care is taken to avoid excessive injury, the type of preparation we employed may retain its viability and its irritability for several hours. Soon after isolation, however, the cut muscle fiber loses the power to transmit a stimulus from one region to another (cf. Brown and Sichel, 1936). In other words, the fiber usually responds only at the point at which it is stimulated. Our data are, therefore, concerned mainly with this type of response and should accordingly be interpreted from this standpoint.

In order to obtain a quantitative measure of the irritability or excitability of the individual fibers, their threshold to electric stimulation was determined. In every case the threshold voltage was that voltage to which a 4-microfarad condenser had to be charged and which, when discharged through the electrodes, would produce the smallest observable response in the fiber. The electrodes were fine silver wires, coated with AgCl and kept at a distance of 10 mm. from each other. They were not allowed to touch the fiber; but an attempt was made to keep the same relation between electrodes and fiber in any given experiment, in order to minimize the effect on the threshold of varying the resistance between the electrodes.

In our measurements, obviously, it is not possible to determine what proportion of the stimulating current passes through, and what portion passes around, the fiber. This is a difficulty that is attendant on practically all measurements of threshold to electric stimulation. It becomes particularly serious if one attempts to compare threshold measurements made in solutions of widely different conductivities. Thus, for example, the threshold of a muscle fiber immersed in sugar solution may be greater or less than that of the fiber immersed in isotonic salt solution, and yet this need not necessarily mean that the fiber is less or more irritable. In interpreting our data, therefore, no attempt should be made to regard the values for thresholds as having an importance as absolute values. Nevertheless, with reasonable caution they can certainly be used for purposes of comparison; and, used in this way, they provide ample proof for our claim that it is the calcium ion, rather than the sodium ion, that is essential for the irritability of the fiber.

Table 2 shows the results of experiments in which fibers were dissected out from mus, cles previously immersed for long periods in isotonic sucrose solution. In the first column the numbers in parentheses identify the individual muscles. Following isolation-some of these fibers were still irritable to the stimulation we had available, although others were not, as indicated by the fact that their threshold is given as being above 105 volts. When these fibers were transferred to isotonic calcium chloride solutions, all but 2 of the fibers showed a marked decrease in threshold. In these 2 instances the fibers did not recover their irritability. Immersion in the calcium chloride solutions was brief, for the fibers live only a short time in such solutions (Heilbrunn, 1940).

It might be suspected that the marked drop in threshold on transfer to the calcium chloride solution was due to the increased conductivity of the salt solution. If this were the case, then transfer from sugar solution to solutions of sodium chloride should have a similar effect. But exactly the opposite is true. This is shown by Table 3, in which, as before, the number in parentheses in the first column identifies the individual muscles. In the experiments listed in this table, of 14 cases in which fibers were dissected out of muscles immersed for long times in isotonic sucrose solution, in nearly every instance the fibers were found to be irritable. But when these irritable fibers were exposed even for a brief time to isotonic solutions of sodium chloride, all but 2 of the fibers showed a complete loss of irritability. Following this loss of irritability (in 10 out of 12 fibers), a small amount of isotonic calcium chloride solution was added to the drop of sodium chloride solution in which the fibers were bathed. As a result, there was a sharp drop of threshold

TABLE 2

Fibers Dissected from—	Duration of Immersion of Muscle in Sucrose	Threshold of Muscle before Dissection of Fibers (Volts)	Threshold of Fibers on Isolation (Volts)	Duration of Immersion in $CaCl_2$ (Minutes)	Threshold in $CaCl_2$ (Volts)
Sartorius (2).........	1 hr. 15 min. 1 24 1 24 1 45	66 66 66 66	75 75 45 105	1 1 1 0.25	36 24 24 9
Sartorius (3).........	0 37	3	105	4.5	15
Sartorius (4)*	0 54 2 28	>105 >105	60 >105	1 0.5	2 >105
Sartorius (7).........	2 33 2 49 2 54 3 18 3 51	6 6 6 6 6	>105 >105 36 >105 >105	0.25 0.5 2.75 3.5 1	102 >105 18 30 60

* The behavior of this particular fiber is shown graphically and in more detail in Fig. 1.

TABLE 3

Fibers Dissected from—	Duration of Immersion of Muscle in Sucrose	Threshold of Muscle before Dissection of Fibers (Volts)	Threshold of Fibers on Isolation (Volts)	Duration of Immersion in NaCl (Minutes)	Threshold in NaCl (Volts)	Duration of Immersion in NaCl+$CaCl_2$ (Minutes)	Threshold in NaCl+$CaCl_2$ (Volts)
Adductor magnus....	2 hrs. 12 min.	....	2	3	45	2	30
Sartorius (1).........	3 16	....	39	1	>105	1	>105
Sartorius (2).........	1 06 1 36 1 41 1 47	66 66 66 66	45 75 18 39	1 0.5 0.5 1	>105 >105 >105 >105	0.5 1 0.5 0.75	30 18 45 49
Sartorius (3).........	0 33	3	36	0.25	>105	0.5	60
Sartorius (4).........	2 24	>105	84	0.5	>105	0.5	96
Sartorius (5)*........	3 01	>105	15	0.25	>105	0.75	45
Sartorius (6).........	4 30 4 39	....	105 96	0.5 1	>105 93	1 0.5	>105 45
Sartorius (7).........	3 01	6	105	0.5	>105	4.5	39
Sartorius (8).........	2 23 2 26	30 30	105 2	0.25 1	>105 >105	0.25 2.5	69 10

* The behavior of this particular fiber is shown graphically and in more detail in Fig. 1.

in all but 2 of the preparations. (In these 2 there was no recovery of irritability.) In the case of these fibers dissected out of muscles previously immersed for a long time in sucrose solution, the results show clearly that immersion in sodium chloride, instead of favoring a restoration of irritability, as Overton's views would lead us to believe, actually reduces or destroys irritability. Clearly, it is the calcium ion which is important for the response of these isolated fibers to electrical stimulation. The data presented in tabular form do not give a complete record of changes in threshold in relation to time as well as to changes in medium. Graphic records could be presented for each of the experiments listed; but in the interests of economy, only 4 such graphs are presented (in Figs. 1 and 2). Figure 1 shows the variations in threshold of 2 fibers dissected from sucrose treated muscles and then immersed in calcium of chloride in the one case and in sodium chloride followed by a sodium chloride–calcium chloride mixture in the other. These 2 graphs represent cases indicated by asterisks in Tables 2 and 3.

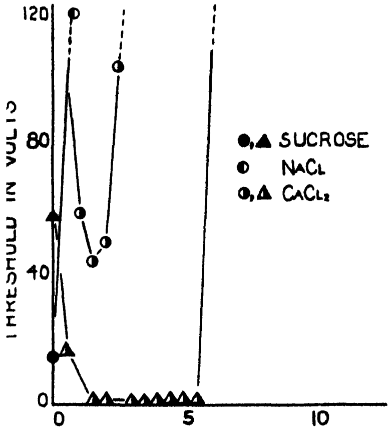

FIG. 1

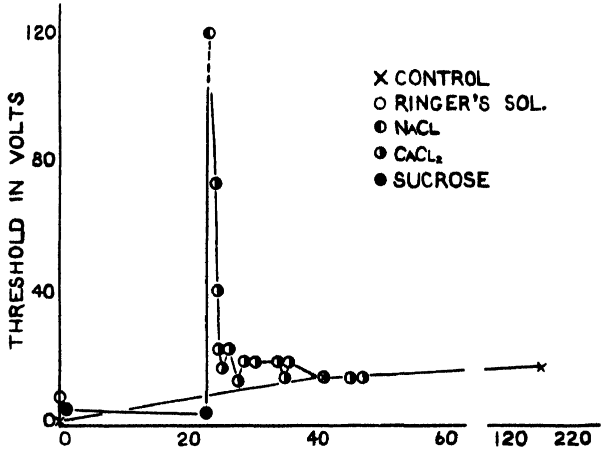

FIG. 2

FIG. 1.—Variations in threshold of a single fiber, isolated from a sucrose-treated muscle, on immersion in isosmotic $CaCl_2$ (triangles) and of a similar fiber on immersion in NaCl followed by $CaCl_2$ (circles). The points on the y-axis give the respective thresholds on isolation of the fibers. The broken lines indicate that the threshold was higher than 105 volts, which was the maximum voltage obtainable.

FIG. 2.—Variations in threshold in response to variations in the medium surrounding a fiber dissected in Ringer's solution, then immersed successively in isosmotic sucrose, NaCl and $CaCl_2$ (circles). Broken lines indicate that the threshold was higher than 105 volts. Crosses show variations in threshold of control fiber in Ringer's solution.

Experiments similar to those outlined above were performed on muscle fibers dissected in Ringer's fluid, then transferred to isotonic sucrose solution and from there into an isotonic solution of sodium chloride (see Table 4). This is essentially a repetition, with isolated fibers, of Overton's basic experiment on whole muscles. In the whole muscles irritability is lost in the sugar solution and regained in the solution of sodium chloride. But Table 4 shows very clearly that for the isolated fibers any loss of irritability in sugar solution is never regained in the sodium chloride solution. Indeed, in 14 out of 17 cases irritability is completely lost in the sodium chloride solution. However, in many cases the addition of a small amount of a calcium chloride solution to the fluid bathing the fibers results in a restoration of irritability; and in every instance but one, the calcium, if it produces any measurable change at all, decreases the threshold of the fiber. Figure 2 shows the variation which a single fiber undergoes on successive immersion in Ringer's solution, isosmotic sucrose, sodium chloride, and calcium chloride, as compared with a control fiber in Ringer's solution.

The experiments reported so far indicate that an isotonic solution of sodium chloride, far from restoring the irritability of muscle fibers previously immersed in sucrose solutions, has the opposite effect. Following decrease or loss of irritability in sodium chloride solutions, the addition of a small amount of isotonic calcium chloride results in an increase in irritability. In these experiments, in order to avoid handling and possible injury of the fiber, the calcium chloride solution was added directly to the drop containing the fiber. One disadvantage of this procedure was that it was not possible to know the exact ratio of calcium to sodium in the resultant solution.

TABLE 4

Threshold in Ringer's Solution (Volts)	Duration of Immersion in Sucrose (Minutes)	Threshold in Sucrose (Volts)	Duration of Immersion in NaCl (Minutes)	Threshold in NaCl (Volts)	Duration of Immersion in NaCl+ $CaCl_2$ (Minutes)	Threshold in NaCl+ $CaCl_2$ (Volts)
6	1	45	0 5	>105	2	36
1 7	3	30	1	>105	2.5	33
5	2 5	27	1	>105	2	30
2	20 5	24	0 5	>105	0.5	60
18	10	30	0 5	>105	0.5	105
15	9	30	1	39	0 5	30
9*	22	4	0 25	>105	4	14
69	1	18	2 5	90	0 5	48
0 5	88	60	1	>105	1	84
45	12 5	33	1	>105	0 5	42
12	1	7	1	12	0 5	21
6	2	54	1	>108	.0 5	108
105	16	57	4	>105	0 5	105
1 5	5	6	0 5	>105	0.5	45
27	1	54	1	>105	1	105
2	27	9	0.5	>105	0.5	30
2	15	27	0.5	>105	0.5	105

* The behavior of this particular fiber is shown graphically and in more detail in Fig 2.

In a final series of experiments (Table 5) fibers were dissected in Ringer's fluid, were transferred to sucrose and then to sodium chloride solution, and finally from the sodium chloride solution to a mixture of sodium and calcium chlorides in the proportion in which they occur in Ringer's fluid. The results were thoroughly consistent. In sucrose the fibers did not lose their irritability, although in general they were less irritable than in Ringer's fluid. However, on transfer to isotonic sodium chloride solution, in all 25 instances a complete loss of irritability resulted. Then on transfer to a solution containing sodium and calcium, a return of irritability occurred.

In summary, it may be stated that all our experiments with isolated fibers show convincingly that the sodium ion has no power to restore or improve the irritability of fibers previously immersed in sugar solution. Indeed, of the three common cations, calcium alone has this power. Our results, therefore, are in direct opposition to the belief of Overton that the irritability of muscle fibers is primarily conditioned by the presence of sodium ion in the medium surrounding them.

TABLE 5

Threshold in Ringer's (Volts)	Duration of Immersion in Sucrose (Minutes)	Threshold in Sucrose (Volts)	Duration of Immersion in NaCl (Minutes)	Threshold in NaCl (Volts)	Duration of Immersion in K-free Ringer's (Minutes)	Threshold in K-free Ringer's (Volts)
1	3	21	0 5	>105	1 0	39
3 5	2 5	12	0 25	>105	1 5	66
18	13 0	30	0 25	>105	0 5	37
2	0 5	1 5	1 5	>105	1 5	75
2 5	0 5	9	0 5	>105	0 5	45
13	1 5	19	0 5	>105	0 75	34
1	1 5	28	0 5	>105	18 0	48
9	2 5	48	0 5	>105	0 75	30
1	2 0	12	0 75	>105	34 75	57
4 5	2 5	8	0 5	>105	1 5	27
3	0 5	12	0 5	>105	1 0	55 5
3 5	24 0	21	0 25	>105	0 5	9
3	2 0	15	0 5	>105	0 5	63
1	1 0	42	0 5	>105	0 25	60
10	1 0	21	0 5	>105	22 5	24
2	1 5	39	0 5	>105	16 5	30
1 5	1 0	30	0 25	>105	0 5	30
3	0 5	10 5	1 0	>105	12 0	27
45	2 0	42	0 5	>105	1 0	36
84	4 5	30	0 5	>105	0 5	30
2 5	0 5	21	0 5	>105	1 0	45
1	1 0	8	0 25	>105	3 25	27
3	2 0	42	0 5	>105	1 0	21
4 5	1 0	24	1 5	>105	1 5	30
3 0	0 25	30	0 5	>105	1 0	75

GENERAL CONCLUSIONS

Overton's experiments are reproducible, and we have no quarrel with them. However, his widely accepted conclusion that the irritability of muscle fibers is primarily dependent on the presence of sodium ion in the surrounding medium can no longer be regarded as valid. When muscles immersed in sugar solution fail to show readily observable response to electrical stimulation, this appears to be in part due to a failure of the electric current to reach individual fibers. (It may also in part be due to a failure of the conduction mechanism within the fibers.) The irritability of the isolated (cut) fiber has been shown to be dependent on the presence of calcium ion. When a fiber is isolated in sucrose solution, irritability is retained for some time, presumably because of the presence of calcium ion in the surface or cortex. Washing of such sucrose-treated fibers with solutions of sodium chloride apparently removes this calcium, for it destroys the irritability. Subsequent restoration of irritability occurs only when calcium ion is added to the medium surrounding the fibers.

SUMMARY

1. As Overton originally stated, frog muscles lose their irritability when immersed in sugar solutions and regain it on being transferred to a solution of a sodium salt.

2. The indirect excitability of the muscle depends on the presence of calcium ion rather than sodium ion. This is in accordance with the opinion of numerous older authors.

3. When muscles have been immersed for several hours in sugar solution, the individual fibers, on isolation, may be found to have retained their irritability.

4. This is taken to indicate that one factor involved in the loss of irritability in sugar solutions is the failure of the sugar solution to conduct the electric current to fibers remote from the electrodes.

5. When muscles are immersed in sugar solutions, irritability to mechanical stimulation is rapidly lost. Such irritability is restored on transfer to solutions of sodium chloride, but typically no restoration of irritability occurs in solutions of sodium citrate or oxalate. The facts with regard to mechanical stimulation are difficult to interpret. It is possible that irritability to mechanical stimulation may depend on the presence of calcium ion in the surface of the fiber, but this cannot be regarded as proved.

6. Experiments with isolated fibers show clearly that, following immersion in sugar solutions, subsequent treatment with solutions of sodium chloride causes only a loss of irritability.

7. Calcium ion increases the irritability of isolated muscle fibers previously immersed in sugar solution, and it restores the irritability of isolated fibers which have become non-irritable following treatment first with sugar and then with sodium chloride solution.

8. The irritability of isolated fibers, and presumably of muscle fibers *in situ*, seems to be dependent on the presence of calcium ion rather than sodium ion.

LITERATURE CITED

ADRIAN, E. D., and GELFAN, S. 1933. Rhythmic activity in skeletal muscle fibres. Jour. Physiol., 78:271.

ASHKENAZ, E. W. 1941. Chemical stimulation of isolated muscle fibers. (In preparation.)

BROWN, D. E. S., and SICHEL, F. J. M. 1936. The isometric contraction of isolated muscle fibers. Jour. Cell. Comp. Physiol., 8:315.

CHAO, I. 1936. Action of calcium and curare on muscular contraction and neuromuscular transmission. Chinese Jour. Physiol., 10:545.

———. 1937. Influence of electrolyte content on muscular contractility, irritability and neuromuscular transmission. *Ibid.*, 12:237.

CICARDO, V. H. 1938. Sensibilidad a las inyecciones intraarteriales de acetilcolina del gastrocnemio del sapo perfundido con soluciones con distintas concentraciones de iones Na, K y Ca. Iones y excitabilidad neuromuscular. Rev. Soc. argent. biol., 14:370.

CUSHING, H. 1901. Concerning the poisonous effect of pure sodium chloride solutions upon the nerve-muscle preparation. Amer. Jour. Physiol., 6:77.

FENG, T. P. 1936. Studies on the neuromuscular junction. II. The universal antagonism between calcium and curarizing agencies. Chinese Jour. Physiol., 10:513.

FENN, W. O. 1931. The oxygen consumption of muscles made non-irritable by sugar solutions. Amer. Jour. Physiol., 97:634.

GELLHORN, E. 1935. The influence of parathormone on the neuro-muscular system: an experimental analysis. Amer. Jour. Physiol., 111:466.

HEILBRUNN, L. V. 1937. An outline of general physiology. Philadelphia.

———. 1940. The action of calcium on muscle protoplasm. Physiol. Zoöl., 13:88.

HEILBRUNN, L. V., and WILBUR, K. M. 1937. Stimulation and nuclear breakdown in the *Nereis* egg. Biol. Bull., 73:557.

HILL, A. V., and KUPALOV, P. S. 1930. The vapour pressure of muscle. Proc. Roy. Soc., London, B, **106**:445.

HÖBER, R. 1907. Beiträge zur physikalischen Chemie der Erregung und der Narkose. Arch. f. d. ges. Physiol., **120**:492.

———. 1926. Physikalische Chemie der Zelle und der Gewebe. 6th ed. Leipzig.

JOSEPH, D. R., and MELTZER, S. J. 1911. Contributions to our knowledge of the action of sodium and calcium upon the direct and indirect irritability of the muscles of the frog. Amer. Jour. Physiol., **29**:1.

KLEEFELD, G. 1913. Contribution à l'étude de la contraction musculaire. (Influence des anélectrolytes, des électrolytes et de la pression osmotique.) Bull. classe sci., Acad. roy. de Belgique, 1913, p. 91.

LAPICQUE, L. and M. 1933. Sur l'action curarisante du chlorure de sodium non balancé. Compt. rend. Soc. biol., **113**:1036.

LILLIE, R. S. 1932. Protoplasmic action and nervous action. 2d ed., Chicago.

LOCKE, F. S. 1894. Notiz über den Einfluss physiologischer Kochsalzlösung auf die elektrische Erregbarkeit von Muskel und Nerv. Centralbl. f. Physiol., **8**:166.

LOEB, R. F. 1924. On the diffusability of the calcium of blood serum through collodion membranes. The effect of sodium chloride and changes in hydrogen ion concentration. Jour. Gen. Physiol., **6**:453.

LOEB, R. F., and NICHOLS, E. G. 1927. Factors influencing the diffusability of calcium in human blood serum. Jour. Biol. Chem., **72**:687.

MARTIN, E. G. 1904. An experimental study of the rhythmic activity of isolated strips of the heart-muscle. Amer. Jour. Physiol., **11**:103.

———. 1906. A study of the relations of the inorganic salts of the blood to the contractions of heart muscle and skeletal muscle. *Ibid.*, **16**:191.

MAZIA, D. 1933. Sodium chloride cytolysis in the sea urchin egg. Anat. Rec., suppl., **57**:39.

OVERTON, E. 1902. Beiträge zur allgemeinen Muskel- und Nervenphysiologie. II. Ueber die Unentbehrlichkeit von Natrium- (oder Lithium-)Ionen für den Contractionsact des Muskels. Arch. f. d. ges. Physiol., **92**:346.

———. 1904. Beiträge zur allgemeinen Muskel- und Nervenphysiologie. III. Studien über die Wirkung der Alkali- und Erdalkalisalze auf Skelettmuskeln und Nerven. *Ibid.*, **105**:176.

POLIAKOFF, S. 1904. Die Erregbarkeit von Nerv und Muskel perfundierter Frösche. Zeitschr. f. Biol., **45**:23.

RAMSEY, R. W., and STREET, S. F. 1938. The alpha excitability of the local and propagated mechanical response in isolated single muscle fibers. Jour. Cell. Comp. Physiol., **12**:361.

SCHEIN, H., and RIESSER, O. 1935. Über die Abhängigkeit der Narkoticawirkung am Muskel von der Calciumkonzentration und die Bedeutung des Calciums für die Erregbarkeit der motorischen Nervenendigungen. Arch. f. exper. Path. u. Pharmakol., **177**:463.

SCHWARTZ, C. 1907. Beiträge zur allgemeinen Muskelphysiologie. I. Über Ermüdung und Erholung von Froschmuskeln unter den Einfluss von Natriumsalzen. Arch. f. d. ges. Physiol., **117**:161.

TAUBMANN, G. 1934. Muskelkalk bei Ca-freier Durchströmung. Arch. f. exper. Path. u. Pharmakol., **174**:476.

TAUBMANN, G., and HIKIJI, K. 1931. Wirkungen vegetativer Ionen und Gifte am Skelettmuskel. Arch. f. exper. Path. u. Pharmakol., **161**:620.

VICTOR, J. 1933. The effects of sugar and electrolyte solutions on the metabolism and irritability of heart muscle. Amer. Jour. Physiol., **103**:620.

THE EFFECTS OF PERIPHERAL INHIBITION ON THE MUSCLE ACTION POTENTIALS OF THE CRAB

(Five figures)

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IT HAS been shown previously (van Harreveld and Wiersma, 1937) that the muscular contractions of decapod crustacean muscles can be inhibited peripherally by the stimulation of certain nerve fibers. In most muscles only one inhibitory fiber has been found, and this fiber can inhibit the contractions caused by any of the from one to four motor fibers which supply the muscle. Recently it has been shown that in the crab, *Cancer anthonyi*, the opener muscle[1] is innervated by two inhibitory fibers both of which inhibit the contractions caused by the stimulation of the single motor axon supplying it (Wiersma, 1941). This situation has opened the possibility of investigating in one muscle the effect of inhibition by two different inhibitory fibers on the muscle action potentials. In the opener of the crayfish, *Cambarus*, where only one inhibitor is present, it has been found that the reduction of the action potentials is by no means necessary for inhibition of the mechanical contraction (Marmont and Wiersma, 1938). Only when the inhibitory impulses arrive at the muscle just before the excitatory ones is a pronounced reduction of the action potentials obtained. This reduction of the action potentials has been termed "supplementary inhibition." No reduction is visible when the inhibitory impulses arrive slightly later (simple inhibition).

One of the two inhibitors innervating the opener in the crab also inhibits the closer, which has a double motor innervation. As a result, the effect of inhibition could be investigated on the two types of action potentials exhibited by a doubly innervated muscle.

METHOD

The technique used to prepare single motor and inhibitory nerve fibers has been described before (van Harreveld and Wiersma, 1936, 1937) and does not need to be repeated here. Only the walking legs of the crabs were used in the present investigation, as claws of *Cancer* are too clumsy to handle, and, furthermore, fatigue occurs much sooner in them. The nerve bundle in the meropodite mainly consists of sensory fiber bundles. The efferent fibers usually are found in two small bundles, one of which contains the two motor axons of the closer muscle, while the other, which is usually Y-shaped, contains the remaining six efferent fibers. Separating the two branches of this Y usually isolates the opener-stretcher motor axon and the opener and the stretcher inhibitors from the two bender motor axons and the "common" inhibitor. This "common" inhibitor has been shown to innervate at least five muscles in the leg, among which are the four distal muscles (Wiersma, 1941). When the inhibitory effect of this fiber on the closer or opener is investigated, it is not obligatory to separate it from the two bender axons, since the action potentials of the bender have been found to be undetectable when leading off from the closer or the opener muscles. For the same reason the action potentials of the stretcher do not disturb the opener action potentials. The stretcher inhibitor usually was left attached either to the opener-stretcher motor axon or to the "true" opener inhibitor. During the experiments with the two inhibitors of the opener the "true" and the "common" inhibitor were always differentiated from each other by testing them against the closer contraction.

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[1] The names "opener" and "closer" will be used in the present paper for the abductor and the adductor muscles, moving the dactylopodite of the chelipeds and the walking legs. The innervation is the same in these legs.

Two methods have been used to obtain differences in the time of arrival of the excitatory and inhibitory impulses at the muscle. The first one has been described before (Marmont and Wiersma, 1938). In this method an induction coil with one primary and two secondaries is used. In this way both fibers are stimulated at the same time, the variable factor used to obtain differences in the arrival time being the distance the impulses have to travel through the nerve fibers. When this method is employed, the stimulating electrodes have to be moved for each difference recorded, and this frequently results in mechanical injury which restricts the area receptive to stimulation. The second method developed to circumvent these difficulties enables a series of two stimuli to be given with a variable interval of time between them. In this method the electrodes were placed at the same level, and moving them was unnecessary. Frequencies ranging between 20 and 100 per second were used. The maximal variation between the two shocks that could be obtained with this apparatus was 8.3 milliseconds; thus, at a basal frequency of 60 per second it was possible to cover the complete cycle. A more detailed description of the apparatus is given in the Appendix, by G. Keighley, who built the stimulator.

The usual procedure employed in the experiments was to stimulate for 6 seconds. During the first 2 seconds of a 6 seconds' stimulation the motor fiber alone was stimulated; during the next 2 seconds the inhibitor was also stimulated (either before or after each excitatory stimulus); and during the final 2 seconds again only the motor axon was stimulated.

The action potentials were recorded by means of a Mathews oscillograph and amplifier. The leading-off electrodes were silver–silver chloride wires which were covered with cotton wool. In some instances records of the mechanograms were simultaneously obtained by attaching a strong-springed auxotonic lever (with a mirror attached) to the dactylopodite.

THE EFFECTS OF THE OPENER INHIBITOR ON THE ACTION POTENTIALS OF THE OPENER MUSCLE

Although no differences in the mechanical inhibition can be detected whether in a series of stimuli the individual impulses in the true opener inhibitor arrive at the muscle immediately before or immediately after the excitatory ones, the difference of the effects on the action potentials is very pronounced. As in the crayfish, inhibitory impulses which are started immediately before the excitatory ones invariably and often markedly reduce the action potentials, and this effect is not perceived when the inhibitory impulses are started after the excitatory ones. The maximal amount of reduction possible is usually obtained when the inhibitory impulses precede the motor impulses by one millisecond (Fig. 1 *A*, *B*, and *C*), using the double-pulse stimulator, or with a distance of 4 mm. between the electrodes, the inhibitory ones in front, using the two secondary coils.

With either method considerable variation was observed between different preparations (cf. Fig. 1, *D*). The range of time-intervals which will cause a noticeable reduction of the action potentials varies also in different preparations. Sometimes the range was limited to about 7 milliseconds (Fig. 1, *A*), while in other preparations it extended over a much greater range (Fig. 1, *B*, *C*, and *D*). The maximum amount of reduction obtainable varied in different preparations, being at most 75 per cent (Fig. 1, *A* and *B*) and at least 50 per cent (Fig. 1, *C* and D). There was no obvious relation between the maximum amount of reduction and the range of time over which this supplemented inhibition could be produced. In the crab, as in the crayfish, the reduction of the action potentials at the onset of a supplementary inhibition is rapid, and in most cases the action potentials soon attain a level which remains fairly constant for the rest of the inhibitory period.

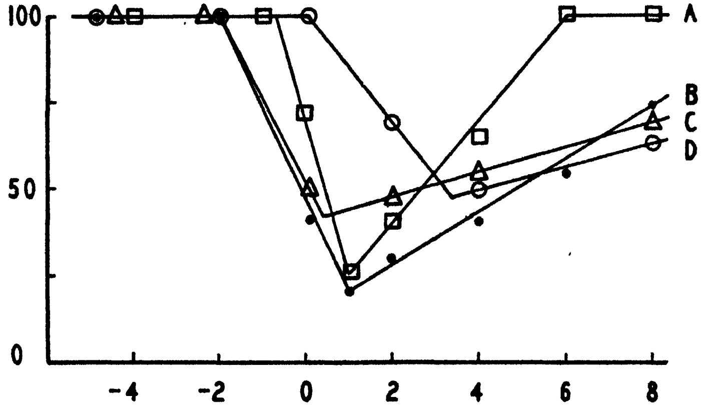

FIG. 1.—Diagram showing the effect of the relative time of arrival of the inhibiting and exciting impulses upon the height of the action potentials in four different preparations. Stimulation frequency of the true opener inhibitor and the opener excitator was 30 per second. The abscissa represents the times in milliseconds that the inhibiting impulses were started in advance of the exciting ones; the ordinate gives the height of the reduced action potentials in percentage of the normal, unreduced height.

On the release from inhibition in the crayfish the reducing effect of the supplementary inhibition disappears immediately after the last inhibitory impulse has produced its effect; but in most of the crab preparations the initial action potentials after release are still considerably depressed, compared with the unreduced height, although even the first uninhibited one always shows a considerable growth (Figs. 2, *B*, and 3, *B*). The return to normal is usually more or less gradual (Fig. 2, *B*). There is thus established during the time of simultaneous excitation and inhibition a state of depression of the height of the action potentials which outlasts the inhibitory stimulation. Although this effect presents itself most clearly after supplementary inhibition, it is also present during simple inhibition. Here it shows itself by a more or less pronounced prevention of the growth of the action potentials during inhibition. In some preparations the growth is totally suppressed during simple inhibition, and in these the stimuli following the release from inhibition cause a pronounced increase in the size of the action potentials (Fig. 2, *A*); in other cases the depression can scarcely be found at all (Fig. 3, *A*). There is an absolute agreement in any one preparation between the effects produced after both kinds of inhibition. If the growth after supplementary inhibition is pronounced, it will also be pronounced after simple inhibition; and if it is small in the first case, it will be too small to be seen in the second. This may be seen in Figures 2 and 3, respectively.

In preparations in which this effect on the growth of the action potentials is very pronounced, the action potentials during simple inhibition are easily distinguished from those which are not inhibited, and the decline of the supplementary inhibited action potentials may go on for a considerable time after the initial rapid reduction. That this effect is present to a certain extent in every case may be shown by the impossibility of obtaining rebound action potentials in the crab. In the crayfish long periods of supplementary inhibition are followed, on release, by action potentials of unusual height. This is explained by continued facilitation and at the same time prevention of fatigue of the action-potential mechanism. In the crab such a rebound was never obtainable, the action potentials never being larger than those before the inhibitory period.

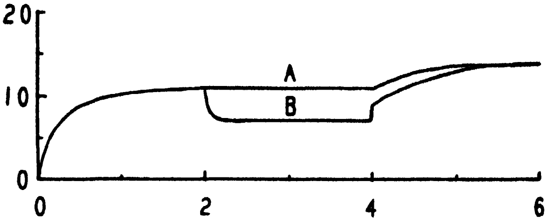

FIG. 2.—Diagram showing simple and supplementary inhibition in a preparation in which the action potential growth is greatly influenced by inhibition. *A* = simple inhibition: during inhibition complete stoppage of growth. *B* = supplementary inhibition: pronounced growth of the action potentials after release of inhibition. Ordinate = height of action potential in millimeters. Abscissa = time in seconds, inhibition from 2 until 4 seconds after start. Curves obtained by drawing a line joining the tops of the action potentials.

After this effect of inhibition on the growth of the action potentials during simultaneous excitation and inhibition had been found in the crab, it was considered likely that inhibitory stimulation by itself would have a very pronounced effect on the growth of the action potentials in a subsequent excitation. Such an after-effect of inhibition has been found to exist to a marked degree in the crayfish. In that animal prolonged stimulation of the inhibitory fiber alone (1 minute or more) is followed by a state in which subsequent stimulation of the motor fiber results in muscle action potentials the growth of which is slower than normal. A series of experiments was completed in the crab in which the inhibitor alone was stimulated for various durations, ranging from a few seconds to several minutes. After the completion of this inhibition, the excitator was immediately stimulated. Remarkably, in none of the experiments was there any pronounced effect on the growth of the action potentials, the differences found always being within the limits of experimental error. Thus there does not seem to be a direct relation between the after-effect as described in the crayfish and the previously mentioned depression of the growth of the action potentials in the crab. It may be mentioned that in both the crab and the

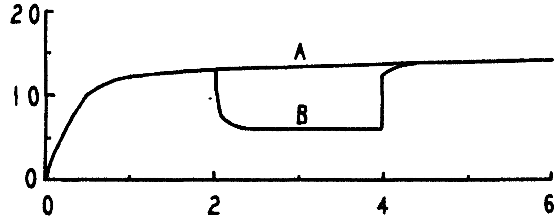

FIG. 3.—Same as Fig. 2, but preparation shows scarcely any influence of inhibition on the action-potential growth. *A* = simple inhibition: no visible effect. *B* = supplementary inhibition: a slight depression on release, which disappears quickly.

crayfish the effect of a previous period of inhibition makes itself observable by a rather marked depression of the mechanical contraction, which continues for some time after the inhibitory stimulation has been applied.

Since it was possible with the apparatus to give the two stimuli at different frequencies, the influence of different frequencies on the amount of supplementary inhibition could also be investigated. With low frequencies the facilitation of the action currents is less, and thus it is not surprising that the absolute height of the action potentials during supplementary inhibition is smaller at low than at high frequencies of stimulation. However, when the reduction of the action potential expressed as a fraction of the height of the unreduced action potential (this latter factor becomes more uncertain the more the reduction is due to depression of the facilitation) is plotted against the time-interval of the two shocks, the figures show a remarkable constancy in all cases below a frequency of 60 per second (Fig. 4). In the frequencies around 60 "simple" inhibition gradually changes into "supplementary" inhibition, and with still higher frequencies only supplementary inhibition can be produced. There will be, of course, as long as the distance between shocks is not too short, a minimum of reduction at values where the inhibitory impulse is set up just after the excitatory one. The fact that changes in frequency have no influence on supplementary inhibition shows that the facilitation of the inhibitory effect must develop at similar rates to that of the excitatory one. Similar results have been obtained in the opener of *Cambarus* using different frequencies of supplementary inhibition.

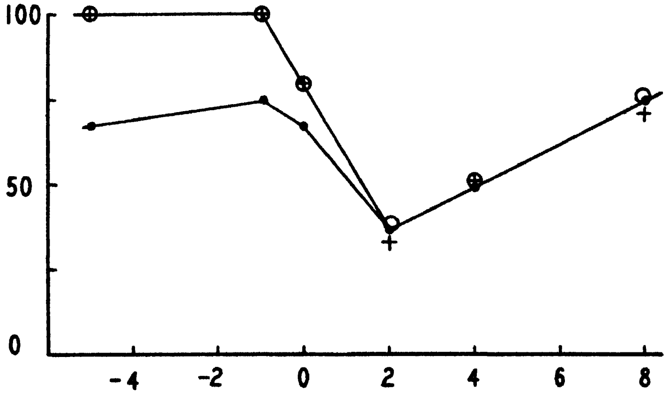

FIG. 4.—Diagram showing the effect on reduction of the action potential of the time of arrival of the inhibitory and excitatory stimuli in one preparation, at different frequencies. Co-ordinates, same as in Fig. 1. ○ represents the values obtained at a frequency of 30 per second; + represents the same at stimulation frequency of both fibers at 50; and ● represents the same at 100 per second.

THE EFFECT OF THE COMMON INHIBITOR ON THE ACTION POTENTIALS OF THE OPENER AND THE CLOSER

The effect of the common inhibitor on the opener action potentials is markedly different from that of the true opener inhibitor. Under no circumstances is there a real supplementary inhibition, for, even in preparations in which the true opener inhibitor causes very marked reductions of the action potentials when the impulses are correctly timed, the effect of this same time-interval or, for that matter, any other time-interval between the impulses of the excitator and the common inhibitor fails to produce any significant reduction. Figure 5, *A* and *B*, shows a comparison of the effects of the common and the true inhibitor in the same preparation.

However, as can be seen in Figure 5, *A*, stimulation of the common inhibitor during excitation is not without some effect on the action potentials. Depending on the preparation, a more or less pronounced stoppage of the growth of the action potentials takes place during this inhibition. It can be shown, however, that this reduction is correlated with the reduction during simple inhibition by the true inhibitor. Preparations in which simple inhibition by the true inhibitor fiber causes a stoppage of growth also present this type of picture when the common inhibitor is stimulated. Since the differences obtained are small, it is impossible to state whether the amount of suppression of growth is the same for stimulation of the two inhibitors. If a difference is present, however, it is very slight. The growth of the action potentials after release from inhibition by the common inhibitor is dependent on the amount of reduction of the facilitation during inhibition, just as is the growth of the action potentials after release from simple inhibition by the opener inhibitor.

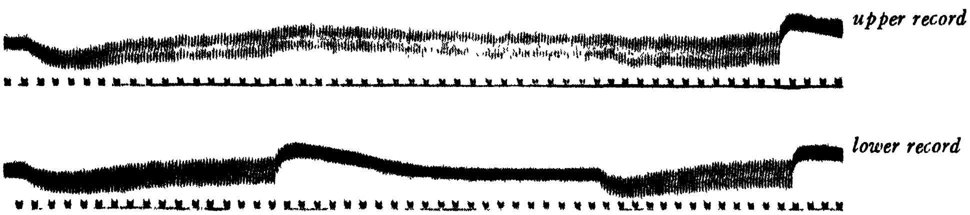

FIG. 5 —Action-potential records of one preparation of the opener of *Cancer* showing the difference of the influence of the common and the true opener inhibitor, when the inhibitory impulses were started 1 millisecond before the excitatory ones. Upper record shows stimulation of the common inhibitor: only very insignificant reduction. Lower tracing gives stimulation of the true opener inhibitor: maximal supplementary inhibition. Time in 1/10 second. Stimulation frequency, 50 per second.

The common inhibitor fiber is only slightly less efficient than the true opener inhibitor in producing mechanical inhibitions. In both cases the contraction is usually completely suppressed by the inhibitory stimulation, and after release from inhibition the contraction is faster than the control contraction. However, in comparison with *Cambarus* this facilitation is not very pronounced. Certain factors, as, for example, fatigue and the length of time during which an opener contraction is facilitated after a foregoing contraction, play an important role in this respect. This makes the experiments less exact than in *Cambarus*, where these factors are of much less influence. It may suffice to state in this regard that, on release, the contraction in *Cancer* may be either faster, equal, or slower than a control contraction given at the same time after the initial contraction as the release contraction. It is likely that slow contraction is correlated with slow growth of the action potentials on release, and vice versa.

The effect of the common inhibitor on the action potentials of the closer muscle resembles that of the common inhibitor on those of the opener but is more pronounced in some preparations. Whereas no real reduction of the opener action potentials has been obtained by the stimulation of this fiber, in a few preparations the closer action potentials may show a noticeable reduction during inhibition. In some cases as much as a 30 per cent reduction has been noticed both in the slow and in the fast action potentials. As a rule, the results of each preparation are uniform. If a reduction is obtained in a preparation, it is observed at all time-intervals and is of equal magnitude throughout. The frequency of stimulation also has no marked influence, although with high frequencies (80–100) the reduction may be quicker and somewhat more pronounced than with low frequencies (20–40). In most preparations, however, the depression of the action-potential growth is only sufficient to slow down or stop the growth, and in some even this effect is hardly visible. In the cases where reduction is present, there is always a pronounced subsequent growth of the action potentials on release, as should be expected from the foregoing results. Thus it appears that the common inhibitor affects the action potentials of both the closer and the opener muscles in the same manner but is more effective on the former.

DISCUSSION

The difference in the reduction of the action potentials of the opener muscle by the two inhibitors is remarkably clear in the curves obtained. It might be argued that the absence of supplementary inhibition by the common inhibitor is due not to a difference in the mechanism but to a special peculiarity of this fiber, which conducts inhibitory impulses to the places where they act in such a way that these different places are reached at various times in relation to those at which the motor impulses arrive. A number of arguments may be raised against this. First, there is no anatomical evidence in the arrangement and divisions of the fibers for any such peculiarity of the common inhibitor. Second, even if such a peculiarity existed, it would not explain the complete absence of any influence of the interval between exciting and inhibiting stimuli on the amount of reduction of the action potentials. Third, pronounced reduction should always be obtained with high frequencies, since the chances of supplementary inhibition at any given point of the muscle then would be greatly increased. However, as high frequencies of stimulation were without much influence on the reduction of the action potentials by the common inhibitor, it must be considered that the common inhibitor is without the ability to give supplementary inhibition.

Another possibility would be that fatigue of supplementary inhibition was very quick on stimulation of the common inhibitor. If this were the case, however, at least a temporary supplementary inhibition would be expected in a fresh preparation.

It is thus concluded that the process of supplementary inhibition and the process responsible for the depression of growth of the action potential, which is not dependent on the time relations, are different. In the crayfish, in which only the first can be found, it has been concluded that inhibition has no influence on the process of action-potential facilitation but that supplementary inhibition results when a block is established in the transmission process between nerve impulse and the process underlying the action potential. This block prevents the fatigue of the action-potential mechanism; and as soon as it is removed, high rebound potentials occur which show that the facilitation process has continued. The absence of such rebound potentials and the fact that in some preparations all intervals of arrival times lead to a stoppage or reduction of the growth point to the conclusion that in the crab inhibition has a depressing effect on the process of facilitation. The magnitude of this effect is, however, different in different preparations and often is very small. Consequently, it is wrong to conclude that in the crab the action of inhibition on the facilitation of the action potentials represents the main factor of inhibition. In the crab, as in the crayfish, the block between the action potentials and the contraction is the main factor in the mechanical inhibition, and in supplementary inhibition it is the block in the transmission process between the nerve impulse and the muscle action potential which always plays the main role. In the majority of cases in the crab it is even true that facilitation of the action potentials goes on notwithstanding inhibition, although the total amount of this facilitation is always at least somewhat diminished during inhibition. As already has been said above, this reduction in facilitation is only of minor significance in the mechanism of inhibition.

It is of interest to note that the opener inhibitor, which must be one of the inhibitors which acts constantly during certain reflexes, produces supplementary inhibition, whereas the common inhibitor, which can play only a very minor part during normal movements, lacks this faculty. It is not likely, however, that there is a relation between the effectiveness of inhibition, on one hand, and the possibility of supplementary inhibition, on the other. Mechanically the inhibition of the opener in the crab by the true opener inhibitor and the slow closer contraction by the common inhibitor have about the same efficiency, whereas supplementary inhibition is found only in the former muscle.

SUMMARY

The influence of stimulation of prepared single inhibitory axons on the muscle action potentials during contraction has been studied in two muscles of the leg of the crab, *Cancer anthonyi.*

In the opener, which receives one exciter and two inhibitors, it was found that the two inhibitors differ in their effects. Stimulation of one of them—the "common" inhibitor—has under no circumstances a pronounced effect on the muscle action potentials. Stimulation of the other—the "true" opener inhibitor—can lead to a considerable (up to 75 per cent) reduction of the potentials if the inhibitory impulses are made to arrive at the muscle just before the excitatory ones. This reduction is similar to, but differs in some respects from, that obtained in the opener muscle of the crayfish.

In the closer, which is innervated by two motor axons and by the "common" inhibitor, the effect of inhibition was studied for both contraction types, which have different action potentials. No reduction of the potentials dependent on the arrival time could be obtained. It is concluded that the "common" inhibitor does not give supplementary inhibition.

A small depression of the growth of the action potentials during any inhibition was found in the crab in contrast with earlier results in the crayfish. It is pointed out that this reduction can have only a minor part in inhibition and that the inhibition of the contraction does not depend in any case on reduction of the action potentials.

A method to obtain two shocks with a variable interval at different frequencies of stimulation is described.

APPENDIX

THE DOUBLE-PULSE STIMULATOR

GEOFFREY KEIGHLEY

The two stimulating pulses with a variable interval between them were obtained by rectifying the output of a stimulator which gave rectangular waves, the frequency and duration of which could be varied independently. The output of this rectangular wave generator was coupled through an audio-transformer with a center tapped secondary to a full-wave rectifier consisting of two triodes. The two outer taps of the secondary were tied directly to the plates; the center tap was divided into a circuit of two legs; and each of these led through a potentiometer to the cathodes. The grids of the two tubes were tied to the plates, the filaments heated by a storage battery. At the beginning of each rectangular wave applied to the rectifier there is a pulse in one direction which passes through one rectifier tube; at the end of each rectangular wave there is a pulse in the other direction which passes through the other rectifier tube. The output is taken from the common terminal of the two potentiometers (the center tap of the transformer) and the two movable taps; the pulses appearing between this common tap and the two variable ones are of the same polarity. The interval of time between the two pulses depends on the duration of the rectangular wave applied to the rectifier, as they are the resultants of the on-off portions of this wave; the interval between each pair of pulses is set by the frequency of the applied rectangular waves. The two potentiometers control the strength of each pulse separately.

LITERATURE CITED

Harreveld, A. van, and Wiersma, C. A. G. 1936. The double motor innervation of the adductor muscle in the claw of the crayfish Jour. Physiol., **88**:78.

———. 1937. The triple innervation of crayfish muscle and its function in contraction and inhibition. Jour. Exper. Biol., **14**:448.

Marmont, G., and Wiersma, C. A. G. 1938. On the mechanism of inhibition and excitation of crayfish muscle. Jour. Physiol., **93**:173.

Wiersma, C. A. G. 1941. The inhibitory nerve supply of the leg muscles of different decapod crustaceans. Jour. Comp. Neurol., **74**:63.

MODIFICATION OF DEVELOPMENT IN THE SAND DOLLAR BY NaCNS AND Ca-FREE SEA WATER[1]

(Thirty-four figures)

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Hopkins Marine Station and Wayne University

IN RECENT years the dynamics of echinoderm development has attracted the attention of such noted investigators as Runnström (1914, 1915, 1917, 1918, 1933, 1935), Hörstadius (1935, 1937, 1939), Lindahl (1933, 1936), von Ubisch (1925, 1929), Child (1915, 1916*a*, 1916*b*, 1929, 1936*a*, 1936*b*, 1940), and many others. The Runnström school at Stockholm has been inclined to look upon the pattern in the early development of the echinoderm egg as the result of an interaction of two opposed material gradients. According to these workers, the animal gradient, extending from the animal to vegetal pole of the egg, tends to ectodermize the larva, while the opposing gradient, from the vegetal to animal pole, tends to entodermize the larva.

Child, on the other hand, has shown with the aid of vital dyes a reduction gradient from the animal to vegetal poles in the early stages. This, together with numerous experiments on larval modifications, has led to the belief that a polar activity gradient may be the primary developmental pattern. He suggests (1936*a*) that the different fields (such as the entoderm field of the late blastula) arise through a process of partial physiological isolation from the primary, or polar gradient.

The work here undertaken deals with the modification of development with NaCNS and Ca-free sea water as test agents in an attempt to throw additional light on the subject of early developmental patterns.

MATERIAL AND METHODS

Only the eggs of the sand dollar, *Dendraster excentricus*, were used in these experiments. Since the work was carried out during the height of the breeding season (June–August), 95–100 per cent fertilization was the rule when a few drops of sperm suspension were added to eggs freshly taken from ripe females. The cultures consisted of approximately 200 eggs in 150 cc. of solution. They were kept in covered finger bowls on a water table. With all tests were run parallel control lots in normal sea water. The eggs were always washed after fertilization, and when removed from the test solutions, by pipetting them through several changes of sea water. During the course of the experiments the temperature varied from 13° to 18° C.

In making up the solutions of NaCNS, Lindahl's method (1933) was followed. This consisted of adding various quantities (5–50 cc.) of the agent in isotonic strength (0.54 M) to 100 cc. of Ca-free or normal sea water. Ca-free sea water was made up, with slight modifications, according to Herbst's formula.[2]

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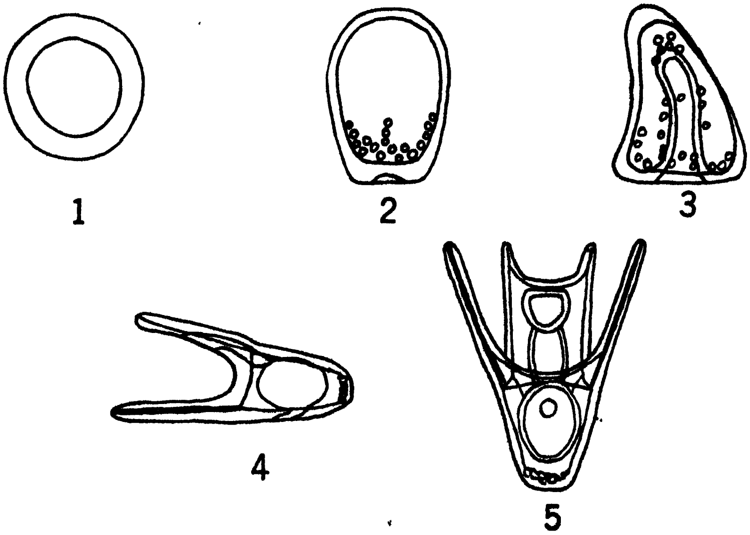
[1] The writer wishes to express his appreciation to Dr. W. K. Fisher, director of the Hopkins Marine Station, Pacific Grove, California, for the laboratory space and facilities which made this work possible. To Dr. C. M. Child the writer is especially indebted for many valuable suggestions and criticisms during the entire course of the work.

[2] Distilled water, 10,571 cc.; NaCl, 307 gm.; KCl, 8 gm.; $MgSO_4$, 66 gm.; $NaHCO_3$, 2 gm.

NORMAL DEVELOPMENT

In most respects the early development of the sand dollar parallels that of the sea urchin, but there are some differences (Child, 1940). For comparative purposes certain of the normal stages are described briefly in the following paragraphs.

The fertilization membrane normally appears within a few minutes after fertilization, and cleavage takes place within this membrane. Cleavage is total and very similar to that of the sea urchin. By 7–8 hours after fertilization the middle blastula stage is reached (Fig. 1). Here the cells are quite small, the epithelium is smooth, and the wall of the blastula is still fairly thick. Cilia are beginning to appear at this stage, and the embryo may be revolving within the fertilization membrane. By 12 hours after fertilization (Fig. 2) most of the embryos are top-swimming, mesenchyme has appeared, and the first signs of gastrulation are noticeable. Except for the extreme vegetal region the walls of the blastocoel have thinned considerably.



FIGS. 1–5.—Normal development of the sand-dollar larva from 7 to 60 hours after fertilization at a temperature of approximately 15° C.

At 17 hours after fertilization bilaterality begins to be evident with a flattened ventral and a rounded dorsal surface. At 24 hours (Fig. 3) bilaterality is distinct, the long archenteron is being directed toward the ventral surface, the oral lobe is directed ventrally, and from a ventral view the first indications of the coelomic pouches may be seen. At this stage the earliest indications of the anal arms, which arise laterally in the posterior or anal regions of the ventral surface, may also be found.

By 45–48 hours after fertilization the larva has become a fairly well-developed pluteus (Fig. 4, lateral aspect). The anal arms are quite long, while the oral lobe has developed arms and become narrower. The skeleton, which was first noted as minute skeletal rods at about 28 hours, is fairly well developed at this stage. A stomodaeum had appeared at approximately 30 hours. At 60 hours (Fig. 5, anal view) the anal and oral arms are longer, and the oral lobe is narrower than in the 45–48-hour larva. The mouth is large and, together with the gut, is usually undergoing rhythmic contractions. At this stage, if no feeding is undertaken, the animals begin to die.

EXPERIMENTAL

1. *The effect of NaCNS on developmental pattern with treatment before fertilization.*—Twenty-four experiments were performed in which unfertilized eggs were submitted to concentrations ranging from 10 to 50 cc. of isotonic NaCNS per 100 cc. of Ca-free sea water for 12–24 hours before fertilization. The eggs were then fertilized and allowed to develop in normal sea water. Parallel experiments were run with normal and Ca-free sea water alone.

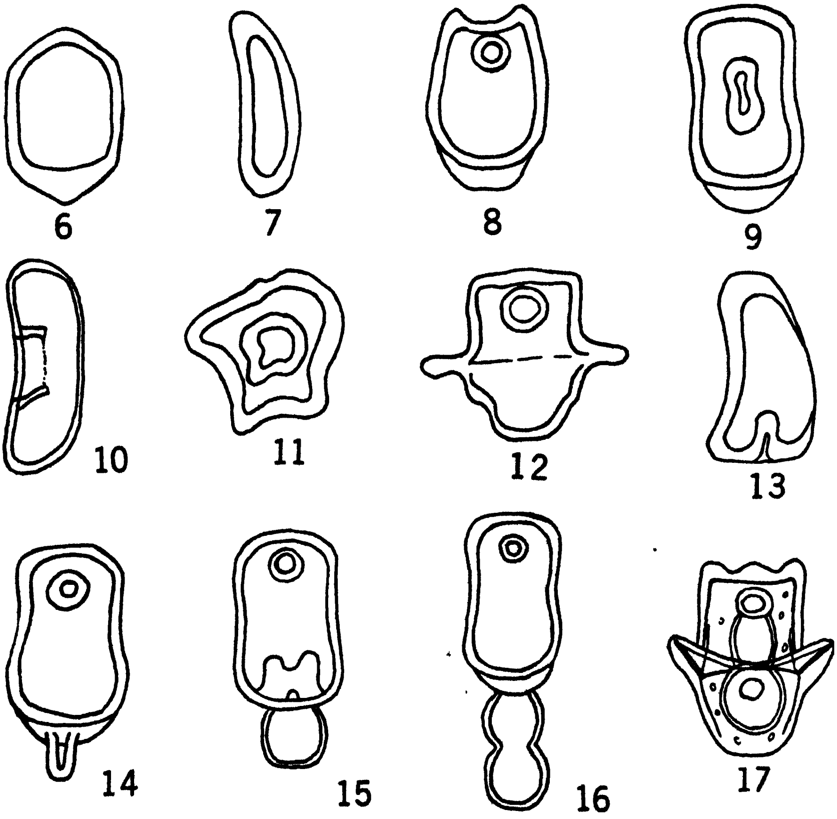
The most interesting results were obtained when the unfertilized eggs were exposed for 12 hours to 20 cc. of isotonic NaCNS plus 100 cc. of Ca-free sea water. Through this treatment the rate of cleavage was retarded when the eggs were returned to normal sea water and fertilized. Observations of such cultures 24 hours after fertilization showed that 30–40 per cent of the living embryos had failed to gastrulate or give off mesenchyme into the blastocoel (Figs. 6 and 7). Most of these mesenchyme-free forms showed considerable movement, as well as some degree of ventrodorsal flattening (Fig. 7). By 60 hours after fertilization most of these forms were still without mesenchyme or gut (Figs. 8–12) and appeared as transparent, pigmentless forms without skeleton but in many cases taking on the general shape of a pluteus (Fig. 12). These larvae may be said to be completely "ectodermized." They usually developed a stomodaeum which opened into the cavity of the blastocoel. In some cases the stomodaeum was considerably enlarged and occupied a central position on the ventral surface.

A number of the forms appearing in the test cultures were only partly ectodermized. Some of these developed a very small evaginated or invaginated gut but no mesenchyme (Figs. 13 and 14), while others developed a diminutive gut with a bit of mesenchyme. In a number the gut appeared late in development as an evagination from the presumptive anal region of a mesenchyme-free larva (Figs. 15 and 16). In some there was a partial invagination of such an evaginated gut (Fig. 15).

Many forms which invaginated but had less mesenchyme than normal developed in these cultures. These developed into plutei showing less differentiation of the oral lobe, shorter anal arms, less skeleton, and less pigment than the normal pluteus (Fig. 17). But not all of the eggs submitted to the test conditions resulted in ectodermized larvae. Some developed into normal plutei, while others showed *excessive* mesenchyme and gave other indications of inhibition, such as short anal arms and failure of the oral lobe to differentiate. There was an occasional exogastrula, and some of the eggs developed no farther than mesenchyme-filled blastulae.

In general, it was found that as the concentration of the test solution and the exposure time were increased the percentage of ectodermized forms decreased and more inhibited forms with surplus mesenchyme appeared. Concentrations of 40 cc. of isotonic NaCNS per 100 cc. of Ca-free sea water for 18 hours and 50 cc. of isotonic NaCNS per 100 cc. of Ca-free sea water for 12 hours preceding fertilization gave no mesenchyme-free forms but chiefly inhibited larvae with excess mesenchyme. In these concentrations many of the eggs failed to cleave or showed aberrant cleavages.

Eleven experiments were performed in which unfertilized eggs were submitted to various concentrations of NaCNS in normal sea water. If the concentration was high enough and the exposure time sufficient, the eggs showed a retardation of cleavage rate and the development of inhibited forms with surplus mesenchyme when fertilized and returned to normal sea water. No ectodermized forms were produced, while many eggs failed to cleave at all or cleaved aberrantly with this treatment. Eggs exposed to Ca-free sea water alone before fertilization and then returned to normal sea water for fertilization and development behaved little differently from the controls. Many experiments showed that eggs from different females behaved somewhat differently in that certain lots could be fertilized following longer periods after removal from the ovary than others. In general, it was found that eggs showed approximately 60 per cent cleavage if fertilized 24 hours after being removed from the ovary.

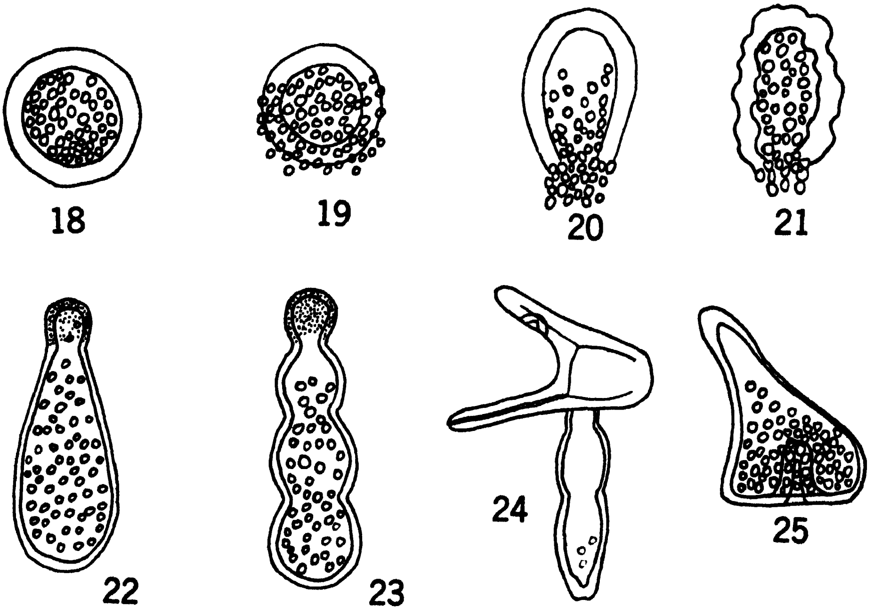


FIGS. 6–17.—Ectodermized forms of the sand-dollar larva resulting from treatment of the unfertilized egg with NaCNS in Ca-free sea water.

2. *The effect of exposure to NaCNS in normal sea water after fertilization.*—Twenty-six experiments were carried out in which fertilized eggs (1–2 cells) were submitted to solutions of NaCNS in normal sea water. In most cases the eggs were washed and returned to normal sea water after a few hours' exposure to the test agent.

When the concentrations were low (5 cc. of 0.54 M NaCNS plus 100 cc. of sea water) and exposure continuous, blastulae appeared which were entirely, or almost entirely, filled with cells (Fig. 18). Approximately 80 per cent of these invaginated, although the process was considerably retarded. When left in this solution, development seldom went past an inhibited gastrula stage. The remaining approximate 20 per cent developed into entexogastrulae. Return to sea water after 50 hours in the test solution resulted in slight recovery of the oral lobe, gut, and ventral side.

When the concentrations were high (40 cc. of 0.54 M NaCNS plus 100 cc. of sea water), the effect of the agent was much more noticeable. Development was much slower, and many aberrancies in the cleavage pattern were noted. Cytolysis was common.



FIGS. 18–25.—Entodermized and recovery forms resulting from treatment with NaCNS in normal sea water after fertilization.

After 12 hours in this solution the developing forms appeared as thick-walled blastulae with cells escaping internally and externally from almost all surfaces (Fig. 19). If the eggs were left in this solution, they usually cytolyzed within 24 hours. In all probability they had become anaxiate, and ventrodorsality had been obliterated long before this time.

When eggs were treated with less toxic solutions (20–30 cc. of 0.54 M NaCNS plus 100 cc. of sea water) for 24 hours, slow-moving thick-walled bottom forms with cells escaping from the vegetal pole resulted in 65–95 per cent of the cases (Fig. 20). (The remaining forms usually appeared as mesenchyme-filled blastulae.) If left in this concentration, they became highly irregular in form (Fig. 21) and soon cytolyzed. If returned to normal sea water after the 24-hour exposure period, they developed, in the vast majority of cases, into typical exogastrulae, which were usually filled with cells and showed the ectoderm reduced to a radially symmetrical knob (Figs. 22 and 23). Such extreme exogastrulae may be said to be almost entirely "entodermized."

The treatment with solutions of NaCNS during early development often resulted in considerable recovery of the ectoderm and ectodermal structures on return to normal sea water if the concentrations were not too severe and the exposure period too long. Exposure for the first 24 hours to 10 cc. of NaCNS plus 100 cc. of sea water gave approximately 45 per cent exogastrulae when the eggs were returned to normal sea water. These exogastrulae were different from those produced with the higher concentrations in that the ectoderm was considerably larger and showed considerable differentiation (Fig. 24). Among these were forms with partial entodermal invagination of either the tip of the evaginated gut, indicating differential recovery, or of the basal gut region, indicating the lower susceptibility of that part of the entoderm. With this treatment many forms gastrulated but showed an increased amount of mesenchyme. In a few some degree of differential recovery was evidenced by the early development of an elongated apical region (Fig. 25). In these experiments, as well as in others in which the treatment was not too severe, a number of normal, or almost normal, plutei developed. These were usually top-swimming, while the affected forms were usually confined to the bottom of the culture dishes.

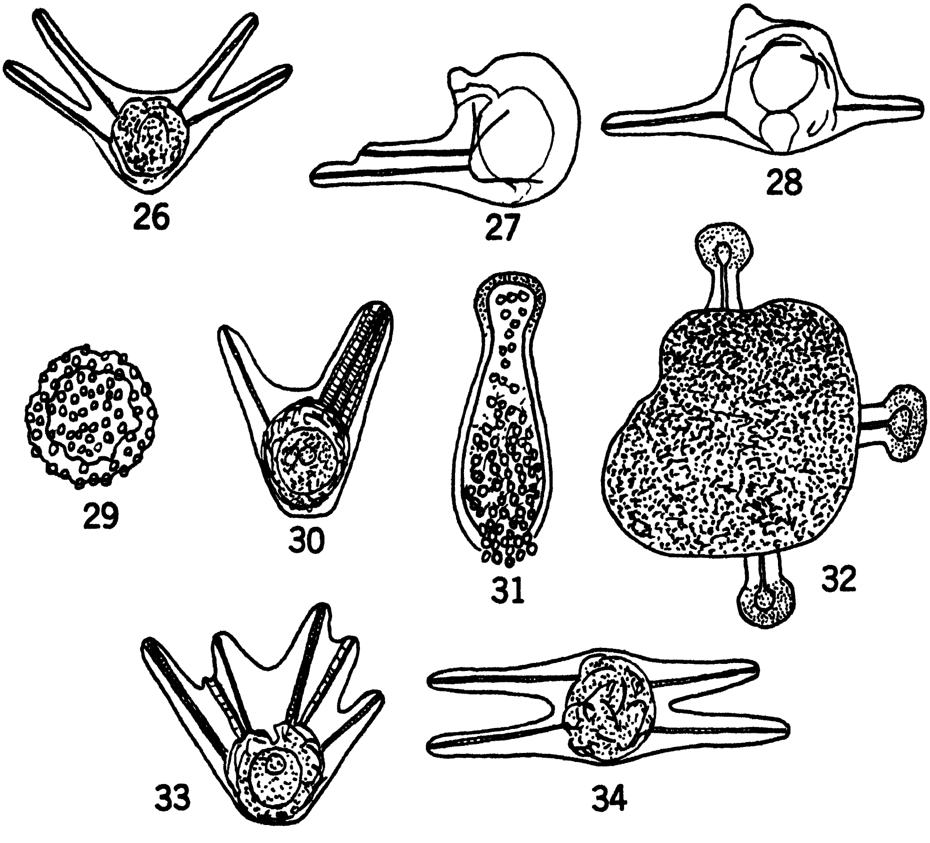
In most of the experiments there were varying numbers of permanent blastulae which were filled with cells or cellular debris and were apparently completely anaxiate, as well as other grossly inhibited forms, depending in a large part on the severity of the treatment. When the initial exposure was 6–8 hours in 40 cc. of NaCNS plus cc. of sea water, only a very few, if any, normal plutei developed. Most of the resulting forms showed an inhibited oral lobe and excess mesenchyme. One or both anal arms were often inhibited; and cell-filled blastulae, as well as exogastrulae, were to be found. Anal arms, when present, were commonly asymmetrical, askew, or at aberrant angles. Aberrant larvae with surplus skeleton were not uncommon.

3. *The effect of exposure to NaCNS in Ca-free sea water after fertilization.*—When fertilized eggs were allowed to develop in solutions of NaCNS in Ca-free sea water for short periods and then returned to normal sea water, the resulting pattern of development was quite different than when the test solution was NaCNS in normal sea water.

An initial exposure of 4 hours to 20 cc. of 0.54 M NaCNS plus 100 cc. of Ca-free sea water showed early blastulae slightly retarded, as compared with controls. Removal to normal sea water at this time resulted, after 45 hours, in plutei which showed marked aberrancies. In general, the oral lobes had developed very little or not at all, while almost all larvae showed an excess amount of skeleton. Many possessed additional anal arms at various angles (Fig. 26), although in some only one large anal arm appeared. When two anal arms developed, they were often fused (Fig. 27), asymmetrical, askew, parallel, or at angles ranging up to 180° (Fig. 28). Few, if any, exogastrulae appeared with this treatment.

An initial exposure of 8 hours to the above test solution produced even more drastic effects. Removal from the test solution at this time showed forms which resembled early middle blastulae but which were irregular in appearance, often ruptured, and were shedding cells from all surfaces (Fig. 29). Many of these forms died; but those that lived developed into larvae showing an excess of mesenchyme, strongly inhibited oral lobes, and often an enormous surplus of skeleton (Fig. 30). Anal arms, when present, were usually asymmetrical, askew, duplicated, or at aberrant angles. Many exogastrulae with a distorted ectoderm appeared with this treatment, while a number of grossly inhibited forms with only stubs of anal arms or no anal arms at all were likewise to be found.

Ten and one-half hours' exposure to this same test solution gave wrinkled, ruptured, middle blastulae which showed no movement and a loss of cells from all surfaces. Many were dead or dying. Fourteen hours after return to normal sea water those that survived the treatment appeared as thick-walled forms losing cells from the vegetal region. These forms developed into exogastrulae with enlarged entoderm and grossly inhibited, radially symmetrical ectoderm (Fig. 31). Often such exogastrulae were stuck together and intimately fused by their entoderms (Fig. 32).



FIGS. 26–34.—Various aberrant forms with distorted patterns resulting from treatment after fertilization with NaCNS in Ca-free sea water and with Ca-free sea water alone.

Fertilized eggs submitted to Ca-free sea water alone for 4 hours developed into normal plutei on return to normal sea water. Eight hours' exposure to Ca-free sea water gave blastulae which were slightly irregular in shape but which developed, for the most part, into normal plutei, although in some the oral lobe was slightly inhibited. Ten and one-half hours' initial exposure to Ca-free sea water had, however, a marked effect. When removed to normal sea water, the forms appeared as broken and ruptured middle blastulae showing loss of cells from all surfaces. They developed into bottom forms with inhibited oral lobes and a surplus amount of skeleton and mesenchyme. Anal arms were often rudimentary or absent but, when present, were usually at aberrant angles, askew, asymmetrical, or duplicated (Figs. 33 and 34). Many of these forms stuck together, creating a mass of skeleton and arms. Few, if any, exogastrulae appeared with this treatment.

DISCUSSION

From these experiments it was quite evident that NaCNS, under the conditions used, behaved as an inhibiting agent. This was shown by the retardation in cleavage rate and the inhibited type of larvae that resulted from continuous exposure. No measurements of respiratory rate were made. Apparently the calcium of normal sea water antagonizes, to some extent, the penetration of NaCNS into the cells. When calcium is removed, it seems clear that the agent not only has a greater effect but is much more rapid in its action.

The ectodermized forms produced here in the sand-dollar larva were less extreme than many of those produced in the sea urchin by Lindahl (1933) and Hörstadius (1935) with NaCNS treatment and the experimental isolation of animal blastomeres but did show more or less "animalization." This same agent, however, when properly applied after fertilization, definitely inhibits ectoderm, and most of the larvae develop toward entoderm and mesenchyme. The production of such different larvae with exposure to the same agent before and after fertilization presents an interesting problem.

Lindahl (1936) maintains that before fertilization NaCNS acts directly in increasing or extending the animal gradient of the egg. He fails to consider, however, the possibility that such ectodermization or animalization may be a secondary effect or recovery on return to normal sea water and of the intense activation following fertilization. In this connection it cannot be emphasized too strongly that in our experiments thiocyanate preceding fertilization retarded cleavage and that, at best, only part of the eggs developed into highly ectodermized forms, the maximum being 30 to 40 per cent. If ectodermization or animalization were a direct effect of NaCNS, we should expect that with increase in concentration or exposure period above that at which ectodermization appeared the percentage frequency would increase to approximately 100 per cent, as in exogastrulation by LiCl and various other agents. This does not happen, however. The maximum frequency of ectodermized forms occurs with concentrations and exposure periods not much above those producing little effect, and with further increase ectodermization is progressively replaced by differential inhibition. In view of this evidence the possibility that ectodermization results from recovery, rather than as a direct effect of the thiocyanate when the eggs are treated before fertilization, appears highly probable.

Exposure of unfertilized eggs to NaCNS in Ca-free sea water apparently results in a general depression or slowing-down of physiological processes, the apical or more active region being retarded to a greater extent than the less active vegetal region. Such differential inhibition may be sufficient to decrease the apical dominance to such a degree that the axiate pattern is partly obliterated. With the stimulation to increased activity, resulting from return to normal sea water and fertilization, the lower levels of the gradient, being wholly or partly physiologically isolated, may recover and develop in the same, or almost the same, way as higher levels. In other words, the whole egg may now develop in a manner similar to the original apical portion. Since the apical region normally gives rise to ectoderm and ectodermal derivatives, the whole egg may now give rise to ectoderm alone, with neither mesenchyme nor gut developing. Such a reconstitution results from an increased scale of organization (see Rulon and Child, 1937), which reaches its peak in forms with a large and centrally placed stomodaeum.

The treatment of fertilized eggs with NaCNS for certain intervals and with certain concentrations results in forms (chiefly exogastrulae) which are not very different from those produced with lithium and other agents (Child, 1940) and lend themselves to a similar interpretation. When the early cleavage stages are exposed to NaCNS, the more active animal pole seems to be primarily inhibited to a greater extent than the vegetal region. The lowered dominance, together with a direct inhibiting action of the agent, may be factors which permit cells to escape from the vegetal region with greater facility than in normal development. Exogastrulae with an extremely reduced ectoderm and enlarged entoderm are not entirely the result of an evagination of the original entoderm. As in the case of Child's lithium-treated larvae, the original entoderm may be partly or completely broken down into mesenchyme-like cells which are lost to the outside or passed into the blastocoel. The large entoderm present may be the result of the inhibiting action of the agent which has depressed the basal levels of the prospective ectoderm to the physiological level of prospective entoderm. Such entodermized ectoderm may be considerably increased in size as a result of recovery on return to normal sea water. Although Child (1940) obtained as high as 100 per cent exogastrulae with lithium treatment, he also noted that Janus green and even crowding tended to entodermize the larvae of certain echinoderms. This, together with the data showing extreme entodermization with NaCNS, does not support the view of the Runnström group that lithium has a specific entodermizing action.

If the exposure time is short or the concentration of the agent is low, there may be considerable recovery and reconstitution of the ectoderm. In many cases the pattern of ectodermal development following initial treatment is considerably distorted. This is particularly true if NaCNS is used in Ca-free sea water solution, although proper exposure to Ca-free sea water alone produces distortion in gastrulated larvae. In general, the oral lobe is markedly inhibited, and excess mesenchyme is present. Commonly the anal arms are at various angles, asymmetrical or askew. Larvae may develop one or many anal arms. These various aberrant forms cannot easily be interpreted, although undoubtedly they represent varying degrees of differential inhibition and recovery of the early embryonic pattern. As stated above, many cells are liberated into the blastocoel during or shortly following the exposure to NaCNS. Calcium-free sea water has long been known to permit the escape or separation of cells in the cleavage or blastula stages. If the treatment is severe, those cells liberated into the blastocoel may develop very little or even cytolyze. However, if the treatment is not too severe, those internal cells may form skeleton in abundance. The appearance of a distorted pattern in anal-arm formation may be related to anomalous distribution or mesenchymal (skeletal) elements, as well as to certain incidental factors, such as contact with the substratum or nearness of other larvae, which may affect the extremely labile condition of the pattern at the critical period of recovery.

SUMMARY

1. Treatment of unfertilized eggs of *Dendraster excentricus* with 20 cc. of 0.54 M NaCNS plus 100 cc. of Ca-free sea water for 12 hours, followed by return to normal sea

water and fertilization, results in retarded cleavage and larvae ranging from ectodermized forms (30–40 per cent) without mesenchyme, gut, or pigment to grossly inhibited forms with excessive mesenchyme, short anal arms, and undifferentiated oral lobes. Higher concentrations and longer exposure periods to this agent decrease the number of ectodermized, and increase the number of inhibited, larvae. Fewer ectodermized and inhibited forms result from lower concentrations and exposure periods. Such evidence favors the view that the ectodermized forms produced by NaCNS treatment before fertilization result from recovery following a differential inhibition of the normal axiate pattern.

2. Fertilized eggs treated with 20–30 cc. of 0.54 M NaCNS plus 100 cc. of normal sea water for 24 hours following fertilization and then returned to normal sea water develop into exogastrulae with considerably enlarged entoderm, reduced ectoderm, and excessive mesenchyme in from 65 to 95 per cent of the cases. In these experiments entodermization seems to result from the depression of the more basal levels of prospective ectoderm to the physiological level of prospective entoderm. Such entodermized ectoderm may be considerably increased in size as a result of recovery on return to normal sea water. The increase in what appears to be mesenchyme seems to result largely or wholly from dissociation and passage into the blastocoel of cells from the original entoderm.

3. With varying concentrations and exposure periods fertilized eggs treated with NaCNS in Ca-free sea water and with Ca-free sea water alone may give rise to larvae ranging from cell-filled blastulae to forms with excessive mesenchyme, with inhibited oral lobe, and with anal arms duplicated, asymmetrical or askew. Such aberrant larvae are interpreted as resulting from varying degrees of differential inhibition and recovery of the early embryonic pattern. Distortion of the pattern may be due to such factors as contact with the substratum, anomalous distribution of skeletal elements, or nearness of other larvae.

LITERATURE CITED

CHILD, C. M. 1915. Axial gradients in the early development of the starfish. Amer. Jour. Physiol., 37:203.

———. 1916*a*. Axial susceptibility gradients in the early development of the sea urchin. Biol. Bull., 30:391.

———. 1916*b*. Experimental control and modification of larval development in the sea urchin in relation to the axial gradients. Jour. Morph., 28:65.

-. 1929. Physiological dominance and physiological isolation in development and reconstitution. Roux Arch. f. Entw'mech., 117:21.

———. 1936*a*. Differential reduction of vital dyes in the early development of echinoderms. *Ibid.*, 135:426.

———. 1936*b*. A contribution to the physiology of exogastrulation in echinoderms. *Ibid.*, p. 457.

———. 1940. Lithium and echinoderm exogastrulation: With a review of the physiological-gradient concept. Physiol. Zoöl., 13:4.

HÖRSTADIUS, S. 1935. Über die Determination im Verlaufe der Eiachse bei Seeigeln. Pubbl. staz. zool. Napoli, 14:251.

———. 1937. Investigations as to the localization of the micromere-, the skeleton-, and the entoderm-forming material in the unfertilized egg of *Arbacia punctulata*. Biol. Bull., 73:295.

———. 1939. The mechanics of sea urchin development, studied by operative methods. Biol. Rev., 14:132.

LINDAHL, P. E. 1933. Über "animalisierte" und "vegetativisierte" Seeigellarven. Roux Arch. f. Entw'mech., 128:661.

———. 1936. Zur Kenntnis der physiologischen Grundlagen der Determination im Seeigelkeim. Acta zool., 17:179.

Rulon, O., and Child, C. M. 1937. Experiments on scale of organization in *Euplanaria dorotocephala*. Physiol. Zool., 10:396.

Runnström, J. 1914. Analytische Studien über die Seeigelentwicklung. I. Arch. f. Entw'mech. de Organ., 40:526.

———. 1915. Analytische Studien über die Seeigelentwicklung. II. *Ibid.*, 41:1.

———. 1917. Analytische Studien über die Seeigelentwicklung. III. *Ibid.*, 43:223.

———. 1918. Analytische Studien über die Seeigelentwicklung. IV–V. *Ibid.*, p. 409.

———. 1933. Kurze Mitteilung zur Physiologie dur Determination des Seeigelkeims. Roux Arch. f. Entw'mech., 129:442.

———. 1935. An analysis of the action of lithium on sea urchin development. Biol. Bull. 68:378.

Ubisch, L. von. 1925. Entwicklungsphysiologische Studien an Seeigelkeimen. Zeitschr. f. wiss. Zool., 124:361.

———. 1929. Über die Determination der larvalen Organe und der Imaginalanlage bei Seeigeln. Roux Arch. f. Entw'mech., 117:80.

EFFECTS OF MODIFIED RINGER SOLUTIONS ON HEAD RECONSTITUTION IN DUGESIA (=EUPLANARIA) DOROTOCEPHALA[1]

(Twenty-eight figures)

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IN AN earlier paper on a study of *Dugesia* (=*Euplanaria*) *dorotocephala* with Murray's modified Ringer solution (Watanabe, 1937) it was shown that definite and orderly variations in death frequency of pieces along the axis of body indicate a physiological differential or gradient of susceptibility to lethal action of the solution as with many other agents the disintegration processes in whole animals do. The present paper includes the results of experiments on head reconstitution and is chiefly concerned with the effect of various concentrations of the solution upon the gradient of head frequency. As to the head-frequency gradient in this species, the paper by Child and Watanabe (1935) may serve, to some extent, as a general background for this paper.

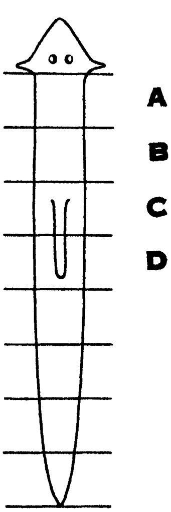

FIG. 1.—Body-levels of pieces used in experiments; each piece is one-eighth of postcephalic length.

The material consisted chiefly of four successive pieces one-eighth of the total postcephalic body length of worms 16–18 mm. in length and representing approximately the anterior zooid. The one-eighth pieces used were designated *A*, *B*, *C*, and *D*, the order being from anterior to posterior levels, as shown in Figure 1. As has often been noted (Child and Watanabe, 1935; Rulon, 1936; etc.), head frequency in reconstitution differs more or less in worms from different localities as well as at different seasons of the year, although it seems not persistently different. Such difference apparently results from difference in environment in nature prior to experimentation. For each series of experiments worms of single collections from single localities were employed.

In order that the animals might be as nearly as possible in the same physiological condition, they were subjected to laboratory conditions with feeding and change of water for at least 2 weeks and then starved 1 week before use, except the worms used in Series 5 and 6. The worms for these series were not fed at all, since, after a trial feeding, they were found to be markedly poor in general condition. The tempera-

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[1] The data presented here, like those in the earlier paper (Child and Watanabe, 1935), were obtained during the years of 1932–34 at the Department of Zoölogy of the University of Chicago.

This investigation was supported in part by funds from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago. This opportunity is taken to acknowledge again the writer's obligations to Dr. C. M. Child and to other members of the staff for the laboratory facilities provided and for many personal kindnesses.

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ture of the laboratory, under which all the experiments described below were performed, was adjusted by thermostat at 19°–21° C.

The solutions and exposure procedure employed were essentially those of the earlier work (Watanabe, 1937). The standard concentration of modified Ringer solution was made up as used by Murray (1928) in planarian tissue-culture experiments and is believed to be approximately isotonic with planarian tissue.[2]

Concentrations used in experiments are given as fractions or multiples of this standard concentration (S) taken as 1. To attain pH 7.6, sodium bicarbonate was added to the solutions of a given concentration; but no attempt was made to buffer the solutions, because any buffer employed, even if not injurious, might introduce effects of the buffer substance. Concentrations and periods of exposure employed are stated in connection with particular experiments.

As suggested earlier (Watanabe, 1937, p. 180), physiological condition of pieces or whole animals in the solutions differs greatly with difference in treatment and depends largely on the relative amount of the solutions to the volume of the tissues and on the frequency of renewal of the solutions during exposure. For this reason a uniform procedure was adopted in all experiments: After section in water pieces were rinsed three times with the solution to be used and 20 or 25 pieces from the same level were put together in 200 or 250 cc. of the solution, i.e., 10 cc. for each piece, without renewal during exposure (see Watanabe, 1937, p. 181). After exposure and three washings pieces remained in water approximately 15 days before head forms were recorded.

The planarian head forms classified by Child (1921)—normal, teratophthalmic, teratomorphic, anophthalmic, and acephalic—constitute a graded series and a convenient basis for distinction of the different degrees of head reconstitution. They have been so often described and figured that further description is unnecessary here (see Child and Watanabe, 1935, and literature cited there). As regards normal heads, however, one point is to be noted. Although the normal head in nature is triangular with two eyespots and two lateral lobes or auricles, in this paper heads with two symmetrical eyespots are recorded as normal, even though shape of head is not entirely normal, in consequence of secondary modifications. The "head-frequency index," a numerical value indicating degree of head development in a given lot of pieces from a particular level, was determined by the formula previously used.[3]

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[2] The standard concentration is as follows:

NaCl . .	2.5 gm.
$CaCl_2 + 2H_2O$ . . .	0.25
KCl . . .	0.05
Distilled water to 1,000 cc.	

[3] Head-frequency index $= \dfrac{(100n_5 + 80n_4 + 60n_3 + 40n_2 + 20n_2 + 0n_0)}{N}$. In this formula n–$_5n_1$ represent the numbers of pieces which develop normal, teratophthalmic, teratomorphic, anophthalmic and acephalic forms, respectively; and n_0, the number of dead pieces. The total number of pieces tested in the lot or series of lots is indicated by N. The numbers 100, 80, 60, 40, and 20 are arbitrary numerical coefficients assigned to the different head forms. As has been pointed out (Watanabe, 1935*b*, p. 136), dead pieces (n_0) are included in this formula because under certain conditions frequency of deaths preceding reconstitution shows a differential distribution in relation to body-level, and it appears desirable to include the total number of pieces in the record. However, definite information concerning head frequency can be obtained only with experimental conditions that give few or no deaths. With high percentages of deaths, head-frequency indices cannot be obtained. The data on head frequency in this paper

HEAD FREQUENCIES OF NORMAL WORMS IN RINGER CONCENTRATIONS ABOVE AND BELOW STANDARD

In *D. dorotocephala* head frequency at a given body-level decreases with decrease in length of pieces; and in pieces below a certain length the frequency decreases from anterior to posterior level of the anterior zooid. In one-eighth pieces head frequency decreases from almost 100 in *A* to a low level in *D*. But, under experimental conditions, these head frequencies are altered in a definite way, with axial differentials. The following data were obtained with an agent not previously used for this purpose and provide further evidence for the orderly relations between concentrations or intensities of agents and the head-frequency gradient.

Series 1. Head frequencies with 14-day exposure to concentrations above standard.—Concentrations from 1 S to 2 S, with controls in well water, were used. In concentrations above 1.9 S total deaths exceed 5 per cent at each level.

As shown by the graphed data of Figure 2, head frequency increases with increase in concentration at each level, except at the *A*-level, at which it is nearly 100 in 1.0–1.8 S and in well water. The increase in head frequency at 1.8 S solution (*P*, Fig. 2) is greatest at *D*-level, next greatest at *C*-level, and least at *B*-level. In consequence of this great increase in head frequency at levels *C* and *D* the gradient of head frequency in 1.8 S solution is distinctly less steep than in 1.0 S or in the controls. In higher concentrations an axial differential in total deaths appears, decreasing posteriorly from the *A*-pieces. This is similar to the decrease in susceptibility of intact animals to many gradually lethal agents.

Series 2. Head frequencies with short-time exposures to concentrations above standard.—Concentrations used were 2.5 S and 3.0 S. In the experiment with 3.0 S solution the pieces were exposed 0, 1½, 3, 4½, and 6 hours, respectively.

Figure 3 shows that head frequency at each level increases with increase in period of exposure up to 3 hours, most in *D*-pieces, very slightly in *A*-pieces. With 3 hours' exposure no deaths occur, but with longer exposures deaths exceed 5 per cent and clearly show an anteroposterior differential. With 6 hours' exposure there were many deaths, most of them without head reconstitution within 48 hours after exposure.

Similar results were obtained with 2.5 S solution. Up to 9 hours' exposure, head frequency in *A*-pieces shows very slight decrease; but in *B*-, *C*-, and *D*-pieces it increases with increase in period of exposure. The 2.5 S solution is much less lethal than the 3.0 S. With 13½ hours' exposure deaths appear in *A*- to *C*-pieces, while in *D*-pieces no deaths occur. With 18 and 27 hours' exposure total deaths for each level exceed 15 per cent and show a beautiful anteroposterior differential.

Series 3. Effect of 14-day exposure to concentrations below standard.—Concentrations used were 0.01 S, 0.05 S, 0.1 S, 0.2 S, 0.4 S, 0.6 S, 0.8 S, and 1.0 S, with controls in well

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were obtained from experiments in which total deaths were less than 5 per cent. With so few deaths, the index, whether based on total number or on living pieces, is practically the same.

It has been pointed out in earlier papers that this formula provides merely a rough and simple means of comparing head frequencies in relation to body-level, length of piece, and environmental conditions and is not based on statistical methods. However, the graphs resulting from application of statistical methods to planarian head frequencies by W. A. Castle (1940) are in general almost identical with those plotted from head-frequency indices. In fact, Castle points out that the method of indices appears in most cases to be entirely adequate for distinguishing differences in head frequency which are not obscure. It has, of course, not been used for any other purpose.

water. Results are graphed in Figure 4. Head frequencies at the concentrations below 0.2 S are not given in this figure. Total deaths in these solutions were more than 5 per cent.

As shown in Figure 4, head frequency at 0.4–1.0 S solutions is always highest in *A*-pieces and decreases posteriorly, and in general it increases as concentration decreases. The difference in head frequency between 0.4 S (*N*) and 1.0 S (*K*) is greatest in *D*-pieces, next greatest in *C*-pieces. less in *B*-pieces, and least in *A*-pieces.

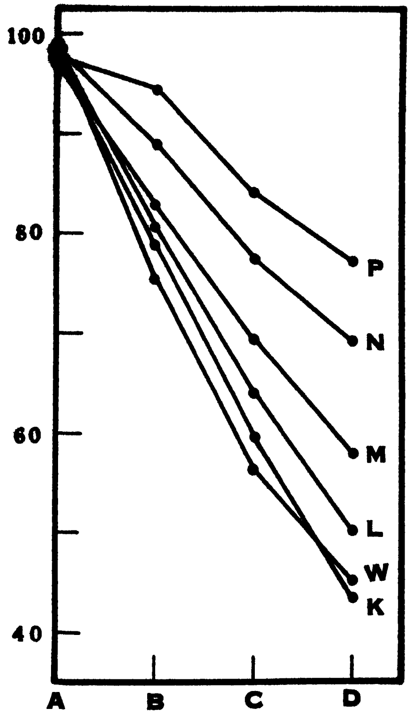


FIG. 2

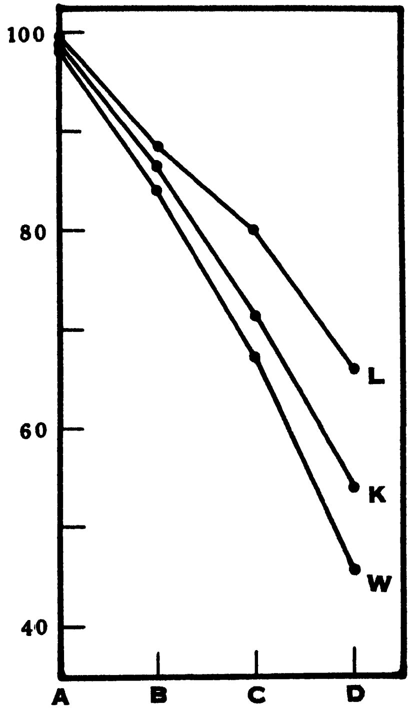


FIG. 3

FIG. 2.—Head frequencies of one-eighth pieces with 14 days' exposure to standard and higher concentrations. *W*, control in water; *K*, 1 S; *L*, 1.2 S; *M*, 1.4 S; *N*, 1.6 S; *P*, 1.8 S. Ordinates are head-frequency indices; abscissae, body-levels. Data are averages of 5 lots, each of pieces from 20 worms for each concentration.

FIG. 3.—Head frequencies of one-eighth pieces in 3 S concentration with different short exposure periods. *W*, control, water; *K*, 1½ hours; *L*, 3 hours. Data are averages for 5 lots, each consisting of 20 worms for each period of exposure.

Series 4. Effects of short-time exposures to concentrations below standard and to distilled water.—Solutions 0.05 and 0.1 S and distilled water were used in this series. To 0.05 S solution the pieces were exposed 0, 9, 18, and 27 hours, respectively; and to 0.1 S solution the pieces were exposed 0, 12, 24, 48, and 72 hours, respectively. Total deaths did not exceed 5 per cent at any level in 0–18 hours' exposures to 0.05 S; and in 0–24 hours' exposures, to 0.1 S. In both concentrations results were very similar as regards relation of head frequency to level and steepness of the gradient curve to exposure period. Figure 5 represents the results of exposure to 0.05 S. The slight differences in *A*- and *B*-pieces are of doubtful significance, but head frequency increases in *C* and still more in *D* with increase in exposure period. Distilled water was also tested as the extreme of dilution. To bring it to pH 7.6 a minute quantity of sodium bicarbonate was added, as in preceding experiments. With 6 hours' and longer exposures all pieces died, and with 3 hours' exposure so many died that the data have little or no value.

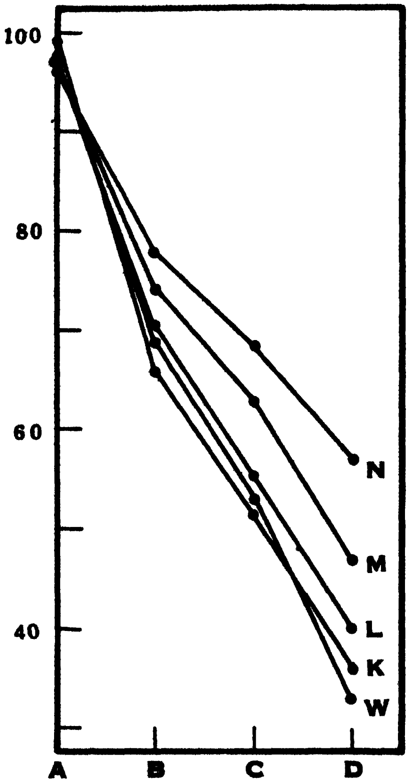


FIG. 4

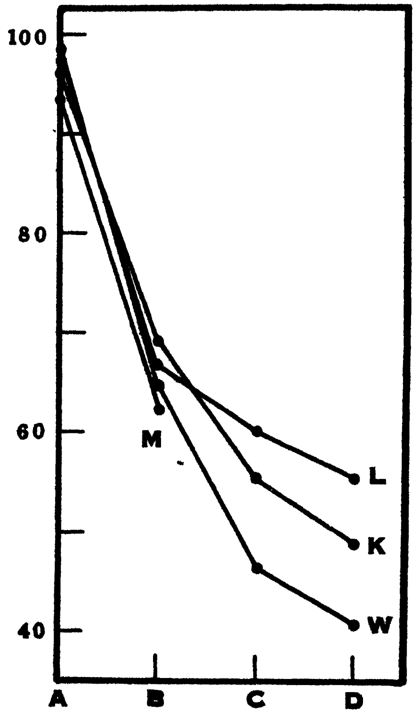


FIG. 5

FIG. 4.—Head frequencies of one-eighth pieces with 14 days' exposure to standard and lower concentrations. *W*, control, water; *K*, 1 S; *L*, 0.8 S; *M*, 0.6 S; *N*, 0.4 S. Data are averages for 5 lots, each of pieces from 20 worms for each concentration.

FIG. 5.—Head frequencies of one-eighth pieces with short exposures to 0.05 S. *W*, control, water; *K*, 9 hours; *L*, 18 hours; *M*, 27 hours. Data are averages for 3 lots of 25 pieces for each level for each period of exposure.

ANOMALOUS HEAD FREQUENCIES OF CERTAIN STOCKS

In certain stocks of worms from Rockford, Illinois, collected in winter and early spring of 1934, an extraordinarily high head frequency and a high bipolar frequency were observed. Head frequencies in normal and some experimental environments are given below. Data on bipolar heads will be reported in a later paper.

The worms of these stocks were evidently not in normal condition: many individuals disintegrated under laboratory conditions, especially after feeding, and pieces showed exceedingly high susceptibility to Ringer concentrations above standard. But these anomalies represented a temporary condition, because the head frequency, biaxial frequency, and susceptibility in worms of other collections from the same spring-fed stream were usually almost the same as those in worms from other localities.

Series 5. Head frequencies of abnormal worms in 14-day exposure to concentrations above standard.—Figure 6 shows the results with 14 days' exposure to 1.0–1.8 S concentrations.

As the graphed data show, the head frequencies of the controls, W, are exceedingly high in this series as compared with those in the preceding series: they are all nearly 100. Even in *D*-pieces the index is 92.5. In Ringer, head frequency does not increase but decreases almost uniformly at all levels with increase in concentration. In 1.9 S solution the total death frequency in pieces from this stock was two to three times that in the pieces from the corresponding levels of worms used in Series 1; and in 2.0 S solution all pieces died in this stock, while in Series 1, from 49 to 82 pieces out of 100 were still alive after 14 days' exposure.

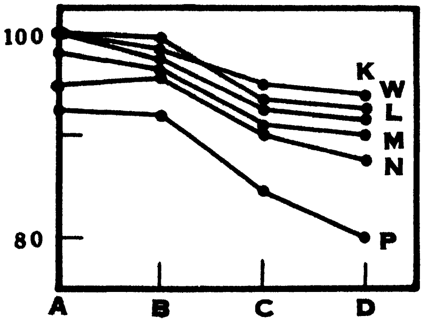


FIG. 6

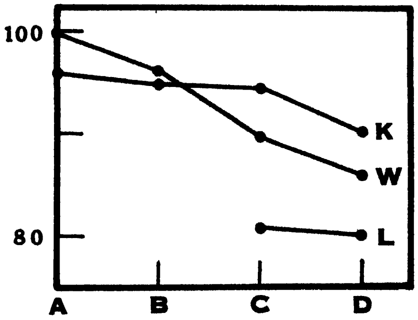


FIG 7

FIG. 6.—Head frequencies of one-eighth pieces from abnormal worms with 14 days' exposure to various concentrations. *W*, control, water; *K*, 1 S; *L*, 1.2 S; *M*, 1.4 S; *N*, 1.6 S; *P*, 1.8 S. Data are averages of 2 lots of 20 worms for each concentration.

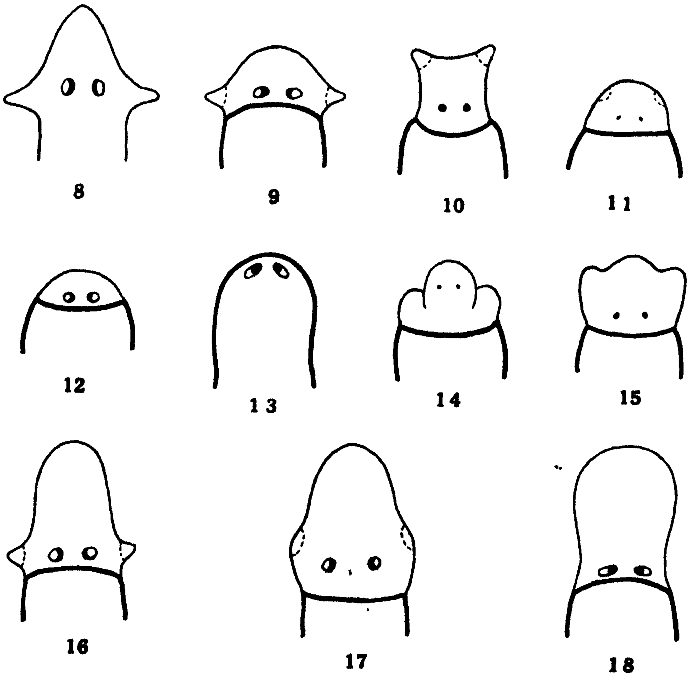
FIG. 7.—Head frequencies of one-eighth pieces from abnormal worms in 2.5 S. *W*, control, water; *K*, 3 hours; *L*, 6 hours. Data are averages of 2 lots of 25 worms for each period of exposure.

Series 6. Head frequencies of abnormal worms in short-time exposures to concentrations above standard.—In this series 2.5 S and 3.0 S solutions were used. General relations of exposure period and head frequencies of the pieces from different levels were nearly the same in both solutions, so that the results with 2.5 S solution only are shown in graphs in Figure 7.

Head frequency of this stock in well water (0 hours' exposure) is still abnormally high, although it is generally a little lower than in the preceding Series 5. The frequency in *C*- and *D*-pieces increases slightly with 3 hours' exposure (*K*) but again decreases at 6 hours' exposure (*L*). At 6 hours' exposure some pieces from *A*- and *B*-levels died, but in *C*- and *D*-levels no deaths occurred.

MEDIOLATERAL DIFFERENTIAL MODIFICATIONS OF NORMAL AND TERATOPHTHALMIC HEADS IN CONCENTRATIONS ABOVE STANDARD

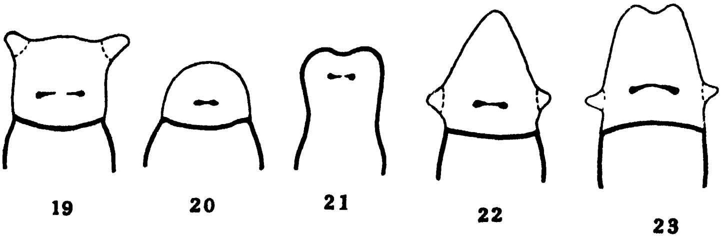
It has been noted in various papers by Child (1916, 1920, 1921) that the different forms of head in regulatory reconstitution of *D. dorotocephala* represent different degrees of differential modification along the mediolateral axis. Similar mediolateral differential modifications of normal and teratophthalmic heads occur with an exposure to Murray-Ringer concentrations above standard. Results of observations are appended here, and characteristic features in modification are shown in semidiagrammatic figures drawn from the observed cases.



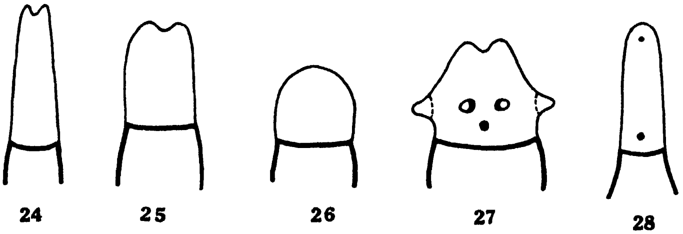
FIGS. 8–18.—Modifications of head form in concentrations above standard. Fig. 8, normal head in nature; Figs. 9–13, mediolateral differential inhibition; Figs. 14–18, mediolateral differential conditioning.

Inhibitory modifications in "normal" heads.—These heads are regarded as normal because eyespots, and presumably ganglia, are normally localized; but other parts of the head may be differentially modified, as compared with the characteristic head form of the species (Fig. 8). With 14 days' exposure to concentrations from 1.9 to 2.2 S the median part of the preganglionic region is more or less inhibited. The preocular length is decreased, and the new head becomes semicircular in shape with two lateral lobes (Fig. 9). With further inhibition the lateral lobes approximate the median line, as in ordinary teratomorphic heads (Fig. 10), and their development is often more or less inhibited (Fig. 11). In extreme inhibition the lobes are entirely absent, and the head is a rounded mass of tissue with two eyespots (Fig. 12). In the higher concentrations, 2.0 and 2.2 S, regeneration of new tissue may be decreased or almost absent, and eyespots develop in the old tissue, as in Figure 13, a *D*-piece in 2.0 S concentration. In these pieces in water, eyespots develop at some distance anterior to the boundary between old and new tissues.

Secondary modifications of "normal" heads due to differential conditioning.—Figures 14–18 show another interesting feature of differential modification in outline of the head in concentrations above standard. In 1.9 and 2.0 S solutions in some pieces no outgrowth occurs during the first week, then after 10–12 days' exposure a new small mass of tissue with two eyespots develops at the median part of the anterior cut surface, and soon after that another small outgrowth appears on each side of this median one (Fig. 14). This form of head apparently represents a mediolateral differential conditioning in reconstitution. The head in Figure 15 is probably also a result of differential conditioning. In concentration 1.9 S these secondary modifications appear less frequently and are less modified than in concentrations 1.6–1.8 S, in which they are often extreme. In some cases the preganglionic region becomes relatively very large and much elongated. In concentrations 1.6 and 1.7 S this overdevelopment may occur without much inhibition of lateral parts (Fig. 16), but in 1.8–1.9 S there is more or less lateral inhibition (Figs. 17 and 18).



FIGS. 19–23.—Modifications of teratophthalmic head in concentrations above standard. Figs. 20–22, mediolateral differential inhibition; Figs. 22 and 23, mediolateral differential conditioning.



FIGS. 24–28.—Secondary modifications of head forms. Figs. 24–26, anophthalmic heads; Figs. 27 and 28, secondarily modified teratomorphic heads.

Differential modifications of teratophthalmic and other heads.—In development of teratophthalmic heads in concentrations above standard two opposed types of mediolateral differential modification appear. With higher concentrations, 1.9 and 2.0 S, inhibitory modifications occur (Figs. 19–21), while with lower concentrations, 1.6 S, differentially conditioned forms develop (Figs. 22 and 23).

In concentrations 1.9 and 2.0 S the new outgrowth is not infrequently without eyespots and different in form from the anophthalmic heads developing in water (Figs. 24–26). These probably involve some differential conditioning.

In 1.6 *S, secondary transformations of teratomorphic heads occur frequently, even* within 2 weeks after section. Figure 27 shows a transformed head without obliteration of a primary median eyespot. The head form of Figure 28 evidently results from secondary median elongation of the anterior outgrowth with a second median eyespot far anterior to the first. Both of these cases undoubtedly represent a differential conditioning in developing teratomorphic heads.

DISCUSSION

It has been shown repeatedly that various inhibiting agents increase planarian head frequency, being most effective on pieces from more posterior levels of the anterior zooid, and that a single concentration of a single agent may decrease head frequency in anterior, and increase it in posterior, pieces.[4] Effects on head frequency of delay of anterior and posterior section (Child and Watanabe, 1935; Watanabe, 1935*b*) have made it evident that the factor inhibiting head reconstitution results from the activation following section at the posterior end of the piece. This inhibiting factor is effective only below a certain length of pieces, which increases from anterior to posterior levels in the anterior zooid and decreases again in the posterior zooid. Moreover, that the inhibiting factor is chiefly or wholly nervous is indicated by the fact that section of nerve cords is almost as effective in increasing head frequency as section of the whole body and by the effectiveness of anesthetics in increasing head frequency (Buchanan, 1922; Watanabe, 1935*b*). There are then two antagonistic factors in determining head frequency in planarian pieces below a certain length—the activity of the cells directly concerned in head development and the effectiveness of the factor inhibiting this activation.

The relation of these two factors differs at different body-levels. At anterior levels the inhibiting factor is effective only in extremely short pieces; consequently, in the pieces ordinarily used for experiment, one-sixth to one-eighth of total postcephalic length of 16–18 mm., anterior pieces have a high head frequency, and any effect of an inhibiting agent will decrease it by action on the cells concerned in head regeneration. In posterior pieces, however, where the factor inhibiting head development is much more effective, an inhibiting agent may inhibit this factor more than it inhibits the head-forming cells and so may increase head frequency; or, since the inhibiting factor is temporary in action, it may be abolished by a short exposure to the inhibiting agent and, after return to water, head development can proceed without inhibition. The various possibilities have been realized in experiment.

It is evident that the planarian Ringer solution acts like an agent inhibiting the inhibiting factor and so increasing head frequency, the amount of increase decreasing from posterior to anterior pieces. Since the solutions have no appreciable effect on head frequency in anterior pieces, they evidently do not greatly inhibit the developmental activities concerned in head regeneration, though they do bring about some degree of differential modification of head form—either differential inhibition or secondary differential conditioning or acclimation.

In the higher Ringer concentrations, intact animals soon become incapable of co-ordinated locomotion or are more or less paralyzed, though muscular contraction is possible. This suggests interference with nervous activity and indicates why this agent is so consistently effective in increasing head frequency in the more posterior pieces and has no significant inhibiting action on head frequency in anterior pieces. With long-time exposures to concentrations above standard the increase in head frequency increases with concentration up to beginning of lethal action (Fig. 2). With short exposures it increases with exposure period (Fig. 3).

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[4] Child, 1916, KCN; Buchanan, 1922, anesthetics; Hinrichs, 1924, caffein; Rulon, 1936, 1937, CO_2, H-ion, organic acids; Miller, 1937, strychnine.

However, with long exposures to concentrations below standard the increase in head frequency increases with decrease in concentration (Fig. 4), and with short-time exposures, with increase in exposure period (Fig. 5), and distilled water is lethal. In these lower concentrations, as in the high, there is no significant decrease of head frequency in anterior pieces, that is, the low concentrations have little or no inhibiting effect on the regenerating tissue. Presumably, therefore, they increase head frequency by inhibiting the inhibiting factor, like the high concentrations; but it appears probable that the high and low concentrations accomplish this result by changes in opposite directions of the electrolyte balance.

In normal worms the variations in head frequency are usually limited within a certain range characteristic for each species.[5] However, head frequency of pieces varies greatly with nutritive condition, physiological age, and environmental conditions in nature prior to experimentation. As shown in the preceding pages, head frequencies of certain worms were extremely high at all levels with very slight gradient. Similar decrease in gradient has also been found in rate of head development in a Japanese planarian. In normal worms of this species, like *D. dorotocephala* and other planarians,[6] the rate decreases from anterior to posterior pieces, but in a certain stock it was found nearly equal at all levels of body (Watanabe, unpublished data).

Reaction of the pieces from all levels of the aberrant stock to Ringer concentrations above the standard resembles that of the most anterior pieces from normal worms in showing no increase in head frequency, like that in pieces from lower levels of normal worms; their head frequencies decrease rather continuously, but slightly, with increase in concentration or exposure time. In these forms with high head frequency the factor inhibiting head development is almost absent, and the effect of the Ringer concentrations is almost entirely on the regenerating tissue; consequently, it decreases head frequency, but, since its effect on this tissue is slight, as shown by Figures 2–5, the decrease is slight.

The mediolateral modifications of head form appear to be either direct mediolateral differential inhibitions or secondary modifications due to differential conditioning similar to those obtained by Child (1921) with ether and alcohol. In the differential inhibitions the median region is most inhibited; in the secondary modifications of differential conditioning it is at first most inhibited, but later it shows relative or absolute overdevelopment. With Ringer these modifications concern chiefly the preocular region. This probably means that inhibition occurs rather slowly and so affects chiefly the later stages of head development rather than eyespots or ganglia; but it is not extreme, and differential conditioning occurs rather rapidly. This is what might be expected with an agent of this sort and is in line with its general effects on head frequency.

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[5] Abeloos, 1930; Sivickis, 1923, 1931; Child and Watanabe, 1935; Watanabe, 1935*b*, 1937, p. 294, footnote; etc.

[6] Watanabe, 1935*a;* Abeloos, 1927, 1930; Sivickis, 1931, 1933; Buchanan, 1933.

SUMMARY

1. Four successive pieces, approximately one-eighth the postcephalic length of animals 16–18 mm. and representing approximately the postcephalic length of the anterior zooid, were used in all experiments.

2. The modified Ringer solution used as agent has no significant effect on head frequency in "standard" concentration, which is believed to be approximately isotonic for planarian tissue.

3. In concentrations above and below standard, head frequency is increased. The increase is greatest at the most posterior level of head development (*D*-pieces), decreases anteriorly from level to level, until at the most anterior level (*A*-pieces) there is no significant increase, and perhaps in some cases a very slight decrease.

4. With long-time exposure (14 days) to concentrations above standard and supposedly hypertonic, head frequency increases with increase in concentration to the beginning of lethal effect. With exposures of a few hours to high concentrations head frequency increases with increase in exposure period.

5. With 14-day exposures to concentrations below standard, supposedly hypotonic, head frequency increases with increasing dilution; but below 0.2 S lethal effect increases with further dilution. With short-time exposures to 0.05 S head frequency increases with increase in exposure period.

6. In general, the modified Ringer acts like other inhibiting agents in increasing head frequency. The experimental evidence indicates that it acts almost entirely by inhibiting the factor, apparently nervous in character, which results from posterior section and inhibits head development. The higher concentrations abolish co-ordinated locomotion and paralyze more or less completely. In the lower concentrations the general effect on head frequency is the same as in the higher, though evidently produced by change in electrolyte balance in the opposite direction.

7. In an aberrant stock showing very high frequency with only slight decrease from anterior to posterior levels, various Ringer concentrations above standard decrease head frequency slightly at all levels. In this material the factor inhibiting head development is almost wholly ineffective, and the slight decrease in head frequency by Ringer represents direct effect on the regenerating tissue.

8. None of the concentrations used have any significant differential effect on the earlier stages of head regeneration but later stages of head regeneration, determining the general form of the head may be differentially modified in two opposite directions in concentrations above standard. In differential inhibition the median preocular region is more inhibited than lateral regions. With secondary modification by differential conditioning this region is relatively or even absolutely overdeveloped in size.

LITERATURE CITED

ABELOOS, M. 1927. La Vitesse de régénération de la tête chez *Planaria gonocephala* Duées. Compt. rend. Acad. sci. Paris, 184:345.

———. 1930. Recherches expérimentales sur la croissance et la régénération chez les planaires. Bull. biol. France et Belgique, 64:1.

BUCHANAN, J. W. 1922. The control of head formation in *Planaria* by means of anesthetics. Jour. Exper. Zoöl., 36:1.

———. 1933. Regeneration in *Phagocata gracilis* (Leidy). Physiol. Zoöl., 6:185.

CASTLE, W. A. 1940. Methods for evaluation of head types in planarians. Physiol. Zoöl., 13:309.

CHILD, C. M. 1916. Studies on the dynamics of morphogenesis and inheritance in experimental reproduction. IX. Jour. Exper. Zoöl., 21:101.

———. 1920. Studies on the dynamics, etc. X. *Ibid.*, 30:403.

———. 1921. Studies on the dynamics, etc. XI. *Ibid.*, 33:409.

CHILD, C. M., and WATANABE, Y. 1935. The head frequency gradient in *Euplanaria dorotocephala*. Physiol. Zoöl., 8:1.

HINRICHS, M. A. 1924. A study of the physiological effects of caffein upon *Planaria dorotocephala*. Jour. Exper. Zoöl., 40:271.

MILLER, F. S. 1937. Some effects of strychnine on reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl., 10:279.

MURRAY, M. A. 1928. The calcium-potassium ratio in culture media for *Planaria dorotocephala*. Physiol. Zoöl., 1:137.

RULON, O. 1936. The effects of carbon dioxide, the hydrogen ion, calcium, and experimental conditioning on reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl., 9:170.

———. 1937. The effects of certain organic acids on reconstitution in *Euplanaria dorotocephala*. *Ibid.*, 10:180.

SIVICKIS, P. B. 1923. Studies on the physiology of reconstitution in *Planaria lata*, with a description of the species. Biol. Bull., 44:113.

———. 1931. A quantitative study of regeneration in *Dendrocoelum lacteum*. Arb. II. Abt. Ungrischen Biol. Forschungsinst., 4:1.

———. 1933. Studies on the physiology of regeneration in triclads. Vytauto disiojo universiteto matematikos-gametos faculteto Darbai (Kaunas), 7:365.

WATANABE, Y. 1935*a*. Rate of head development as indicated by time of appearance of eyes in the reconstitution of *Euplanaria dorotocephala*. Physiol. Zoöl., 8:41.

———. 1935*b*. Head frequency in *Euplanaria maculata* in relation to the nervous system. *Ibid.*, p. 374.

———. 1937. Susceptibility of planarian pieces to various concentrations of Murray-Ringer solution. Sci. Rpts. Tohoku Imper. Univ., Biol., 11:279.

THE ROLE OF A CUT SURFACE IN TUBULARIA REGENERATION

(Five figures)

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IT IS generally recognized that a recently cut surface is prerequisite to regeneration. This paper tells of an attempt to learn the factors associated with a cut surface which evoke the regeneration of lost structures in *Tubularia crocea*.

The first recent step toward a solution of this problem in *Tubularia* was made by Zwilling (1939). He found that not the cutting of tissue but, rather, an unrestricted contact of tissue with sea water was a necessary precursor to regeneration.

It was suggested that the causal agent associating sea water and regeneration might be oxygen. This suggestion was inspired by the knowledge that the oxygen concentration in the range normal for sea water greatly affects the rate of regeneration of *Tubularia* (Barth, 1938*a*). Furthermore, it is shown in the same paper that when the oxygen tension is high the regenerant is long, extending far back from the cut surface. It was suggested that possibly the length of the regenerant is determined by the length of the effective oxygen diffusion gradient, with the oxygen entering primarily through the perisarc-free, cut surface.

With the facts and ideas presented by Barth and Zwilling as a foundation, an attempt has been made to determine some of the factors important in regeneration and associated with a cut surface.

OXYGEN AND THE CUT SURFACE

The first question asked was whether oxygen may be considered to be the stimulus to regeneration. The common method of preventing regeneration in *Tubularia* is to tie ligatures around the cut ends of the stem. If ligatures prevent regeneration by excluding oxygen, regeneration might be expected to occur when oxygen bubbles are added to the coelenteron and the cut ends are ligatured.

Oxygen and air injections were performed under water in a Petri dish. The injection pipette was a glass tube drawn out to a narrow tip, small enough to be inserted in the coelenteron. The pipette was sealed to the dish with paraffin. After the filling of the coelenteron, ligatures were secured about the stem ends with No. 100 cotton thread.

In order to learn whether the effect of oxygen was a specific chemical effect, the experiment was controlled by one group of stems filled with another gas, nitrogen. Another set of controls was filled with oxygen, but the gas was immediately released. The latter are called "empty." Gas disappeared from the gas-filled stems slowly. Usually a small remnant of the bubble was present at the time for primordium appearance.

The experiments are divided into two groups. The stems of one group were kept in running sea water; and those of the other were left in standing sea water, which was changed twice daily. Each group is subdivided into those kept between 10° and 15° C. and those between 18° and 22° C.

The results are presented in Table 1. It may be seen that approximately 20 per cent of the ligatured stems in the running, warm-water group regenerated when filled with air or oxygen. Without added oxygen the percentage of regeneration was much less. The clear difference between the O_2-filled and the empty stems indicates that the latter suffered from a lack of oxygen. This lack was the result of ligatured ends. It is concluded that the open stem end furnishes passage to an amount of oxygen which makes the difference in some cases between regeneration and no regeneration. The cut surface allows greater passage of oxygen to the tissues than occurs when all entering oxygen must pass through perisarc. Enough oxygen can enter through perisarc to keep stems alive for weeks, but a greater supply than this is necessary for regeneration when there is no passage of substances to and from the stem through an open surface.

TABLE 1

COMPARISON OF NUMBERS OF STEMS FORMING PRIMORDIA UNDER VARIOUS CONDITIONS

(All Stems Ligatured at Both Ends)

Condition	Temperature	Total Number	Regenerating Stems	
			Number	Percentage
	Set A. Running Sea Water			
Air-filled	10°–15°	10	0	0
O_2-filled		26	1	4
N_2-filled		12	0	0
Empty		10	0	0
Air-filled	18°–22°	34	7	21
O_2-filled		48	10	21
N_2-filled		40	1	2.5
Empty		124	3	2.5
	Set B. Still Sea Water			
O_2-filled	10°–15°	8	0	0
N_2-filled		5	0	0
Empty		15	0	0
O_2-filled	18°–22°	20	0	0
N_2-filled		20	0	0
Empty		20	0	0

A role other than oxygen passage is also played by the cut surface in regeneration. This may be seen from a comparison of the results obtained when O_2-filled stems were kept in running water with the results obtained when stems were kept in standing water. Twenty-one per cent of the O_2-filled stems regenerated in warm running water. In standing water none regenerated. It appears that the exchange of substances between tissue and water is too slow for regeneration to occur when the water is still. It is fairly certain that the stems had sufficient oxygen. They kept their bubbles for 20 hours or more, and oxygen is necessary for regeneration only during the first 5 or 10 hours of the regeneration period (Barth, 1940c). Moreover, 20 stems were reinjected with oxygen when the first bubble had grown small. Only two of these regenerated. This leaves the possibilities that the stems did not regenerate because other substances entered or left the tissue too slowly. The open end, then, is, in most cases, a necessary passageway not only for oxygen but also for other substances whose presence or absence may inhibit regeneration. The possibility that other substances enter too slowly when there is no open surface has not been explored. An argument that regeneration-inhibiting substances build up when there is no open end is presented in another section of this paper.

The next experiment was designed to show whether regeneration can commence without a region open to sea water and consequently without a region more exposed to oxygen than before the cut. Stems were ligatured for a part of the preprimordium period and kept in standing sea water. The ligatures were then removed from the distal ends. The regeneration times of these stems were compared with those for control stems which had open distal ends from the beginning of the experiment. In order to eliminate the complicating factor of competition by a proximal hydranth, proximal ends were ligatured from the beginning of the experiment on both control and experimental stems. It may be seen from Table 2 that, although distal ends were ligatured for as much as 22 hours, the regenerants which appeared there were only a very few hours behind those appearing in the stems which had been open at the distal end the entire time. Regeneration had started while the stems were ligatured. *Tubularia marina* also starts to regenerate at closed ends when stems are isolated (Morgan and Stevens, 1904). This shows quite strikingly that an increased oxygen supply which results from exposure of an end to sea water is not the stimulus to regeneration. Stems without that exposure to sea water start to regenerate. Distal regions start to regenerate following isolation even without exposed surfaces. An exposed surface is necessary, however, for the continuation of the regeneration. As shown above, one reason for regeneration with an exposed surface is the resulting greater oxygen supply. The experiment just described was conducted in November, and the water temperature was approximately 16° C. Ten times the same experiment was repeated with summer stems at temperatures of 18°–21° C.

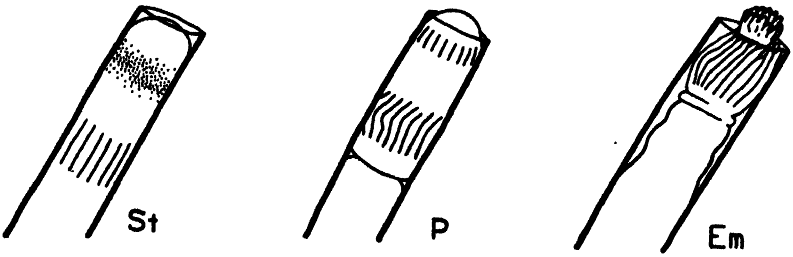


FIG. 1.—Stages in hydranth regeneration at cut end of *Tubularia* stem. *St* = striated stage when a primordium is first visible. The striations are proximal tentacles in the process of formation. The coenosarc has become pink in the region where the distal tentacles will form (indicated by stippling). *P* = pinched stage, when the primordium is partially constricted from the rest of the coenosarc. *Em* = emerging stage when the young hydranth is emerging from the perisarc.

These showed no evidence of having started to regenerate during the period of ligature. However, summer stems did begin to regenerate while ligatured when the temperature of the sea water was reduced to 16° C. Possibly not enough oxygen diffuses through the perisarc from the warmer water. Whatever the explanation may be, the fact is established that regeneration is not always dependent for its start on the increase in oxygen supply which follows cutting. The fact that stems do not complete the process of regeneration when the ends are closed brings us again to another factor associated with a cut surface. Before this other factor is considered, the role of oxygen is explored further.

TABLE 2

COMPARISON OF REGENERATION TIMES FOR STEMS LIGATURED FOR VARIOUS LENGTHS OF TIME*

Number of Stems	Hours Ligatured at Distal End	Hours To Reach Stage	
		Pinched	Emerging
5	0	46	55
5	4	46	56
5	20	48	60
5	0	46	55
5	3	46	57
5	22	50	62
10	21	51	62

* The times recorded are hours from the beginning of the experiment when the stems were isolated The time given to reach a stage is the time for the middle stem of each group to reach that stage An illustration of these stages will be found in Fig 1.

All stems were 20 mm. long and were ligatured at the proximal end throughout the experiment.

Conditions· Still water for all stems ligatured distally until distal ligatures were removed from all stems All the stems were then kept in running water The stems never ligatured at the distal end were kept in running water from the beginning of the experiment. Temperature was 16° ± 2° C.

OXYGEN AS A FACTOR DETERMINING THE LOCUS OF REGENERATION

Regeneration occurs at cut surfaces, and the oxygen supply to the tissues at those surfaces is presumably higher than elsewhere. Does a high local oxygen concentration favor regeneration locally?

Observations on the O_2-filled, ligatured stems in the first experiment suggested the possibility that high local concentrations of oxygen favor regeneration. Only those stems which attained the stage where definite primordia were present are included in Table 1. Many more stems—over half—showed an increase in pink pigment after oxygen injection. A few of the N_2-filled stems also became pink. With some of the O_2-filled stems this pink pigment appeared evenly throughout the stem. In others the increase in color was confined to the distal end. A few stems showed pink around small bubbles of gas which remained. Although definite primordia appeared only once in a region other than the distal, it was considered possible that these local pink areas were young regeneration centers. Some stems were selected which showed localized pink pigment at some point other than the distal. These stems were cut transversely in such a way as to leave the pink portion near the cut surface. The transverse cut was made 24 hours after the oxygen injection and a few hours after the disappearance of the bubble. In a few of these, hydranth primordia appeared in the pink region within 3–10 hours after cutting. Unligatured stems at the temperature of these experiments begin to show primordia approximately 20 hours after cutting. The pink regions may be considered local regeneration centers. It is to be noted that hydranths do not develop in these centers unless the stem is opened.

It is impossible to decide immediately whether oxygen, acting chemically, favors regeneration locally. The oxygen bubble, by blocking the circulation, might have allowed more proximal regions to regenerate. A drop of oil, blocking the coelenteron circulation, has been shown to diminish the dominance of distal over proximal regenerants (Barth, 1938*b*).

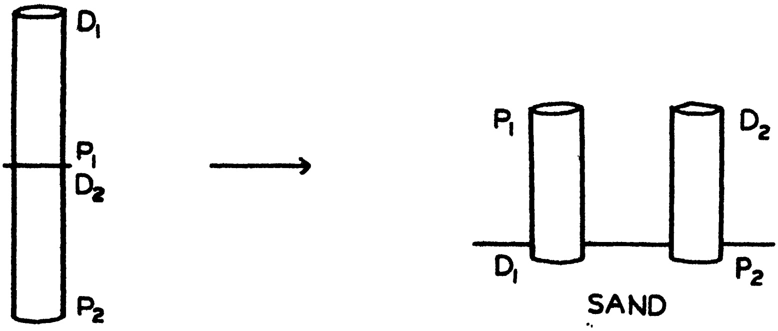


FIG. 2.—The plan for a demonstration of polarity in *Tubularia*. Stem cut into two segments after removal of distal hydranth. The subscript 1 indicates the more distal segment; the subscript 2 indicates the more proximal segment. D and P denote the distal and proximal ends of the segments. Regeneration at D_1 and P_2 inhibited by placing in sand. Adapted from an experiment by Loeb.

A modification of an old experiment by Loeb furnished a test for the hypothesis that bubbles in the coelenteron diminish the dominance of distal over proximal regions. Loeb (1892) found, when *Tubularia* stems were sectioned as in Figure 2 and regeneration at D_1 and P_2 was inhibited, that regeneration was completed at D_2 much sooner than at P_1. Driesch (1899) has shown that more distal stem levels regenerate faster than do more proximal levels. In Loeb's experiments the regenerant at P_1 was drawing upon more distal levels, D_1–P_1, than was D_2, which drew from the region D_2–P_2; yet regeneration at P_1 was slower than at D_2. The two neighboring regions at P_1 and D_2 must have had a similar constitution with respect to the materials which are the physical basis of the observed activity gradient. One region, P_1, however, was drawing, during reconstitution, upon higher gradient levels; and the other, D_2, upon lower, more proximal gradient levels. It is known (Barth, 1938*b*) that a competition between distal and proximal regenerating regions exists, with the distal being favored. This experiment of Loeb appears to indicate that competition still exists between distal and proximal regions even when the inhibition at the distal end is great enough to prevent the appearance of a regenerant there. Although the D_1 and P_2 regions are both inhibited, they still affect the rate of regeneration at P_1 and D_2. The competition of D_1 with respect to P_1 is more effective than that of P_2 with respect to D_2.

If oxygen causes nondistal regeneration by blocking the circulation and thereby the dominance, other gases should do the same. In this experiment three groups of stems were selected. The stems of one group were filled with oxygen, those of another with nitrogen, and those of a third were left without added gas. All stems were ligatured at both ends at the beginning of the experment. After varying lengths of time a transverse cut was made, as shown in Figure 3. The time to reach particular stages was recorded for the regenerants at P_1 and at D_2. The results are summarized in Table 3.

The empty stems of Groups A and B regenerated as expected. The regenerants at P_1 formed much more slowly than those at D_2. Presumably the slow rate at P_1 was caused by the competition of more distal regions. When a gas was added to the coelenteron, either oxygen or nitrogen, the inhibition at P_1 was diminished. This shows that internal oxygen bubbles are unsatisfactory when testing for local chemical effects of oxygen, since they have a physical effect, that of blocking the circulation. Another method was devised for use in study of local oxygen effects.

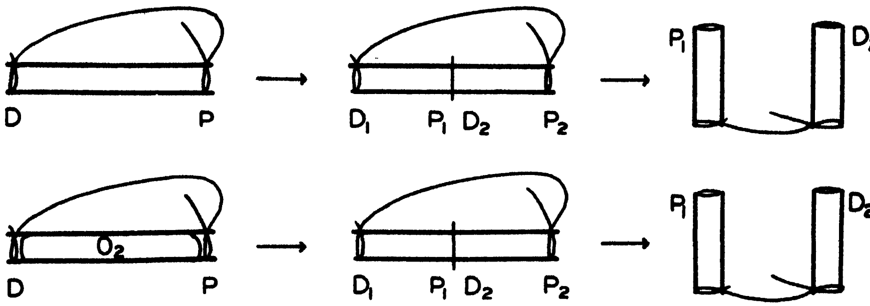


FIG. 3.—Diagram of experiment to test influence of oxygen on competitive behavior of stems. One group of stems ligatured empty, the other group filled with oxygen and ligatured. Both groups cut in middle after oxygen had disappeared from second group. Ligatures at distal and proximal ends connected to allow comparison of regeneration times at D_1 and P_2 in individual cases. Notations as in Fig. 2.

The data of Table 3, although of little value in settling the question whether oxygen is a local stimulant to regeneration, are interesting in other ways. In a few of the cases there was visible evidence that regeneration started in the distal region of empty stems when no cut surface was exposed to oxygen. The fact that P_1 regeneration was extremely slow is considered further evidence that regeneration had started in the more distal regions. Again it is shown that exposure to oxygen cannot be considered the initiating stimulus to regeneration.

The D_1 center, next to the ligature, does not persist as a dominant region. After a cut is made at P_1, a regenerant at P_1 usually dominates, but the effect of D_1 is great enough to retard regeneration at P_1.

None of the stems in Group C of Table 3 give much evidence of the polarity effect. These stems were ligatured for 62 hours before the P_1–D_2 cut was made. Regeneration was more rapid at D_2 in 14 cases, more rapid at P_1 in 7 cases, and the same rate was observed at both D_2 and P_1 in 8 cases. After stems were ligatured for even longer periods—5, 8, 10, 12 days—no difference between the average rates of regeneration at P_1 and D_2

was noticeable. In some stems regeneration was more rapid at D_2; in others a hydranth appeared at P_1 first. The absence of the polarity effect in stems tied off for longer periods is considered an indication that the distal region does not remain an actively competing region when the ends are ligatured. Possibly the fact that almost half of these stems formed buds somewhere between the ligatures while still ligatured is evidence that the distal end no longer dominated. The point of appearance of buds was not restricted to any region of the stem. Some arose near the distal end, some near the proximal, and others in the middle region. Although the distal region no longer actively dominates in stems ligatured for long periods, it can be shown that the gradient structure remains. After the ligatures were cut away and both the distal and proximal ends were exposed to sea water, the distal region developed more rapidly than the proximal, and domi-

TABLE 3

COMPARISON OF REGENERATION TIMES OF DISTAL AND PROXIMAL HYDRANTHS FROM THE SAME STEM LEVEL AFTER INJECTION OF STEM WITH N_2 AND O_2

GROUP	CONDITIONS	NUMBER OF STEMS IN GROUP	TREATMENT	NUMBER OF CASES IN WHICH GIVEN REGION REGENERATED MORE RAPIDLY			REGION[*]	TIME IN HOURS FOR MEDIAN STEM OF EACH GROUP TO REACH GIVEN STAGE	
				D_2	P_1	Same Rate at Both Regions		Striated	Emerging
A......	Standing sea water changed daily; temperature, 19° ± 2° C.; ligatured 26 hours	10	Empty	8	0	2	D_2 P_1	43 66[†]	66 89
		10	O_2-filled	7	1	2	D_2 P_1	37 40	54 61
		10	N_2-filled	6	2	2	D_2 P_1	43 49	49 70
B......	Slowly running sea water; temperature, 17° ± 2°; ligatured 48 hours	5	Empty	5	0	0	D_2 P_1	....	42 58[‡]
		5	O_2-filled	0	2	3	D_2 P_1	48 42	58 56[§]
C......	Slowly running sea water; temperature, 20° ± 1°; ligatured 62 hours	10	Empty	5	2	3	D_2 P_1	31 34	41 44
		10	O_2-filled	4	3	3	D_2 P_1	28 28	36 35
		9	N_2-filled	5	2	2	D_2 P_1	24 29	34 42

[*] Segments lettered as in Fig. 3. Stems 20 mm. in length, ligatured for varying lengths of time, then cut in middle when bubbles were very small or had disappeared.

[†] Three stems were very pink at D_1 ligatures at 69 hours.

[‡] A hydranth formed at the ligatured end D_1 in one case.

[§] Oxygen-filled stems showed several regions of concentrated pink along their lengths.

nance was expressed. These results are similar to those obtained by Peebles (1931) in stems ligatured for several days.

The data of Table 3 may be considered evidence in support of the competition theory of dominance. The gases which block the circulation also decrease the dominance of distal over more proximal regions.

All of the direct demonstrations that dominance is established by way of the circulation have been made by stopping the circulation. The problem has been attacked by another method. This time circulation was permitted, but the tissues between two regions in question were severed. Four groups of stems were prepared. All the stems were approximately 10 mm. in length. The tissue and the circulation between distal and proximal regions were left intact in one group. Stems ligatured in the mid-region comprised a second group. In these both the tissue and the circulation were severed. The stems of a third group had the tissue severed between distal and proximal regions, but circulation continued through the coelenteron between distal and proximal regions. This was done by inserting a short capillary into the coelenteron. The stems were rolled over the bottom of the operating dish, and pressure was applied by forceps in the region of the capillary. This served to force the tissue away from the space between perisarc and capillary. A ligature was then tied around the stem. The ligature prevented reunion of the severed tissue. Circulation, somewhat impaired, persisted for approximately 20 hours through the capillary lumen. It was later blocked by the formation of tissue across the ends of the capillary. The stems of the last group had their circulation blocked by a short glass rod. The tissue was left intact. It may be seen from Table 4 that a distal regenerant usually prevented regeneration in the proximal region when the circulation and the tissue were intact. If both circulation and tissue connections were severed by a ligature as in Group B, regeneration occurred at both distal and proximal ends. Distal regions did not dominate when the tissue was intact but the circulation blocked, as in Group C. Distal regions did dominate when the tissue was severed and only the circulation remained. Therefore dominance is mediated by the coelenteron.

It has been shown that some stems without an open surface do not regenerate because insufficient oxygen is present. An attempt to learn whether local high oxygen concentration plays a part in determining the locus of regeneration was at first unsuccessful. The attempt was unsuccessful because injected oxygen bubbles diminished the dominance of distal regions by blocking the circulation. Local oxygen effects have been studied by other methods.

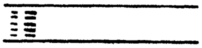
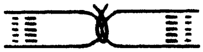
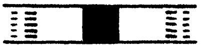
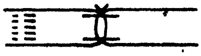
Torrey (1912) showed that when one half of a stem was bathed with boiled sea water and the other half with boiled and aerated sea water the regenerant always formed at the open end of the half bathed with aerated water, whether that end was distal or proximal. Miller (1937) conducted a similar experiment but, instead, bathed half of the stem in sea water saturated with oxygen and the other half in N_2-saturated sea water. The new hydranth always formed on the O_2-bathed end. In these experiments conducted by Torrey and by Miller the end of the stem at which the hydranth did not form was bathed in water practically free of oxygen. The experiments show that regions in which the oxygen concentration is very low do not regenerate. In the present work we are interested in learning whether the greater oxygen supply to the tissues near a cut surface is sufficient to enable that region to be the dominant regeneration center.

The experiment depicted in Figure 4 entails treatment of cut surfaces in several different ways. Here *a* represents cut pieces of stem used as controls, and *b*, *c*, and *d* show stems whose ends were covered with large-mouthed caps. Such large-mouthed caps containing an oxygen bubble were placed over the ends of other stems, as indicated in *e–g*. The O_2-filled caps of *h* and *i* differ from those of *e–g* in that their narrow ends fitted closely over the stems. The stems in the *j*-group were ligatured at the distal end and covered with large-mouthed oxygen caps at the proximal end.

In Figure 4 the stems *b–d* differed from the *e–g* stems in the concentration of oxygen present in their caps. In the *b–d* group hydranths did not form at capped ends. In the *e–g* group, capped with oxygen, hydranths formed at the distal end, no matter whether the oxygen was applied distally, proximally, or at both ends. (Sometimes they also formed at the proximal end, but not with significantly greater frequency than in the controls.) Caps locally inhibit hydranth formation. This inhibition is over ridden when oxygen is present in high concentration. Whatever may have been the mode of action of the caps in preventing regeneration at the distal end, their effect was neutralized by the added oxygen. The oxygen in this case acted locally in allowing regeneration at the near-by surface. It is to be noted, however, that oxygen applied proximally did not enable the proximal region to overcome the inherent dominance of the distal region. In fact, the distal regions of the stems treated proximally with oxygen, *f*, regenerated more rapidly than either the uncapped controls, *a*, or the proximally capped stems, *c*. The *j*-stems, which differed by having a distal ligature, formed no distal hydranths. The stems shown in *h* and *i* differed from *e* and *g* only in that the small open ends of their O_2-filled caps fitted closely over the perisarc. When the tight-fitting caps were used, regeneration was inhibited at the capped end, in spite of the fact that the oxygen bubble was present. This result indicates that a factor other than oxygen was responsible for the inhibition at this end, which was bathed with a very small volume of sea water. The inhibition might result from the depletion of substances obtained from sea water and/or from the accumulation of metabolic substances removable by sea water.

TABLE 4

THE EFFECT ON DOMINANCE OF OBSTRUCTION OF CIRCULATION AND SEVERANCE OF TISSUE

Group	Treatment and Usual Place of Regeneration	Number of Stems	Pinched Primordium at Distal before Any at Proximal	
			Number	Percentage
A		30	28	93
B		28	3	11
C		11	1	9
D		30	20	67

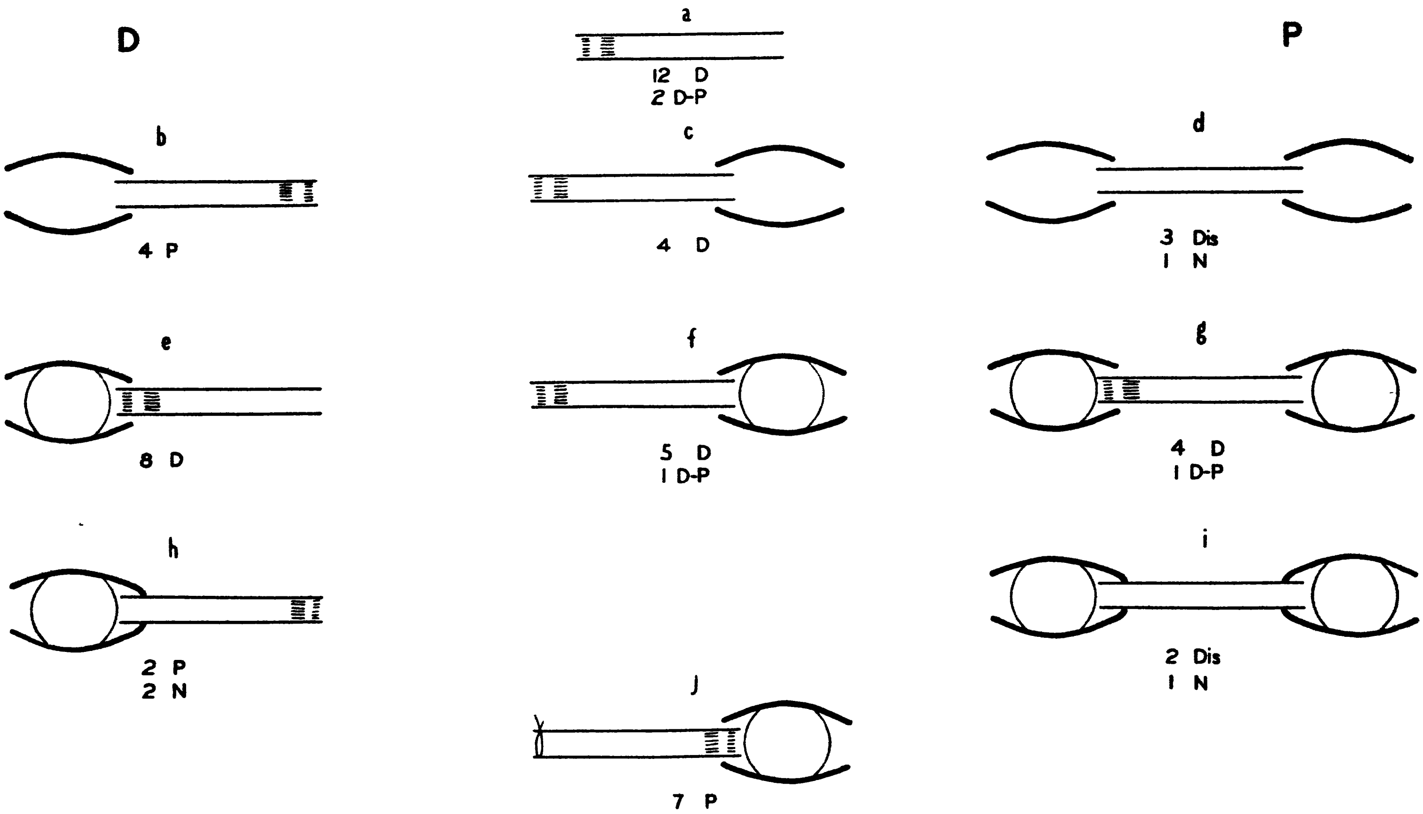


Fig. 4.—Diagram of capping experiments. Position at which regeneration occurred indicated. Distal ends of stems facing left. *D* indicates regeneration at distal end only, *P* indicates regeneration at proximal end only, *D–P* denotes regeneration at both ends. *Dis* = disintegrating coenosarc. *N* = no regeneration. Conditions: Slowly running sea water. Temperature, 20° ± 0.5°. Stems 20 mm. in length.

The results of the experiment shown in Figure 5 are similar to those just described, but the experiment was conducted in a somewhat different way. If the smaller caps, the Figure 5 type, even though they contained oxygen, were left on stems until reconstitution had occurred, the regenerant never formed at the capped end. In the experiment shown, caps were left in place only 19 hours. The position and the stage of regeneration 31 hours after the caps were removed is recorded. It may be seen that the distal regenerants of the formerly O_2-capped stems (*a*) were in the pinched stage, as were those of the untouched controls (*e*). On the other hand, distal regenerants did not appear in the *c*-group, which had been capped but had not received the added oxygen. This is interpreted to mean that a low oxygen supply at one end of a stem in the period before the appearance of primordia inhibits regeneration at that end. There is also the possibility that a cap inhibits during this early period in some way other than by restricting the oxygen supply. If this is the case, the beneficial effect of oxygen can largely neutralize the inhibition of a cap during the early hours of reconstitution. There is some evidence that even the O_2-filled cap inhibits locally, because in *a* of Figure 5 some of the

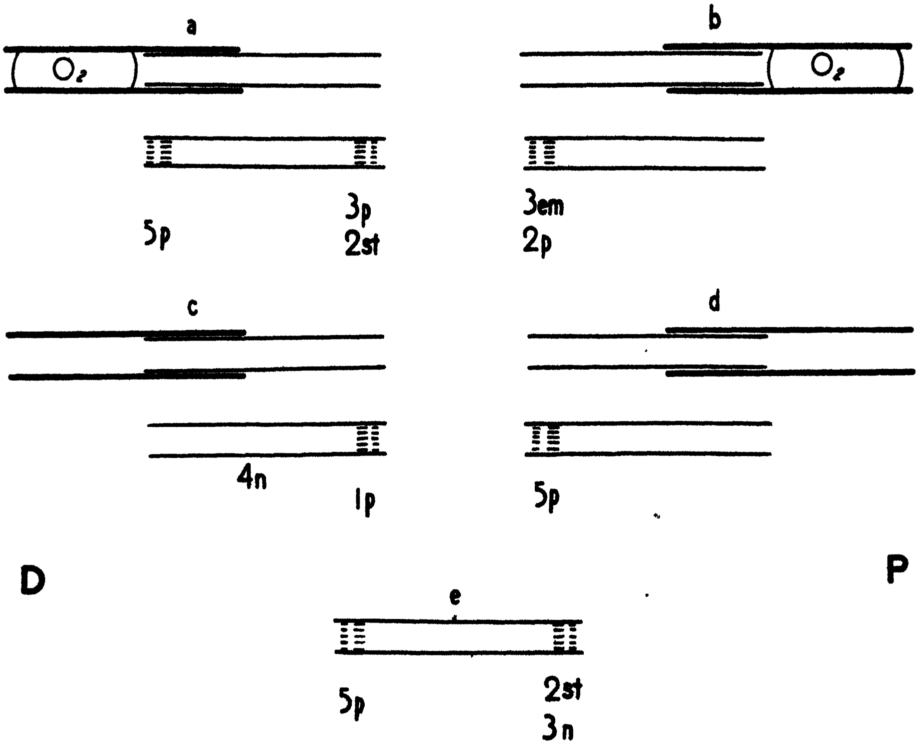


FIG. 5.—Diagram of results of further capping experiments. Stems capped from fourth to twenty-third hour after cutting. The position and stage of regeneration 31 hours after caps were removed is recorded. See Fig. 1 for stages of regeneration. *n* = no regeneration. Distal ends of stems at left. Conditions: Standing sea water. Temperature, 18.5° ± 2°. Stems 25 mm. in length.

distal hydranths had not developed so far as those of *b*. Moreover, proximal hydranths formed on the *a*-stems but not on the *b*-stems which had had proximally placed oxygen caps.

Definite evidence of a locally inhibiting factor other than a low oxygen supply comes from the result that regenerants never formed when the small O_2-filled caps were left in place several days.

The group showing the most rapid development at the distal end was, as in the former experiment, the group which had been treated with oxygen at the proximal end. When much oxygen was available to the stem, the distal region regenerated most rapidly, whether the oxygen had entered from the distal or the proximal end.

Since distal regenerants appeared slightly ahead of controls when oxygen was applied proximally in these experiments with few stems, an experiment was performed with more stems to see if more rapid distal regeneration really follows proximal oxygen treatment. One group of 50 stems was capped proximally with oxygen, as in Figure 4, *f*. Another group of 50 was capped proximally without oxygen, as in Figure 4, *c*. Fifty more stems remained uncapped. Here, again, regeneration occurred at the distal end when the oxygen was added at the proximal end. The proximal region even with added oxygen was dominated by the distal end. Furthermore, the rate of regeneration at the distal end was not decreased when oxygen was applied proximally. The average times to reach the pinched-off stage in the three groups were very close. The uncapped took 31.7 hours; the empty-capped, 31.3 hours; and the O_2-capped, 30.8 hours. The averages are so close and the times for individual stems so variable that it is impossible to conclude from this experiment that oxygen applied proximally causes an increased rate of regeneration in the distal region.

It is concluded from the capping experiments that a cap over a cut surface neutralizes the effect of the cut surface by diminishing the local oxygen supply. However, the effect of added oxygen at a proximal cut surface is not great enough to overcome distal dominance. Evidence that another factor associated with a cut surface also plays a part in determining the place of regeneration has continually appeared.

RELEASE OF INHIBITING SUBSTANCES AT THE CUT SURFACE

It has become evident from the data already presented that the cut surface acts on regeneration in some way other than by allowing rapid oxygen passage. The possibility has been considered that regeneration-inhibiting substances produced in the tissues build up to a level incompatible with regeneration unless they can diffuse away. The primary path of exit is through the open stem end. If regeneration is inhibited by some substance or substances released through the open end to sea water, it should be possible to collect the inhibitor in the water.

Small colonies of approximately two hundred stems were cut into pieces, allowed to heal, washed, and put into flasks containing 250 cc. of sea water. Air was bubbled continuously and vigorously through the flasks. After a time water was removed from the flasks and used as a medium for freshly cut stems. At the same time, control stems were placed in untreated sea water. The experiment was performed 17 times with groups of 40 stems. Every time inhibition by colony water was apparent. In general, the amount of inhibition was proportional to the time the water had been in contact with the colony. In Table 5 the data for groups of stems grown in sea water which had been in contact with a colony for varying times are given. It may be seen that as the time of contact with the colony increased, more stems failed to regenerate, and those which did regenerate did so more slowly.

The data from experiment to experiment is quite variable. Sometimes, for example, 2-hour colony water inhibited but a small percentage of the stems; at other times it almost completely inhibited. However, 12-hour colony water completely inhibited regeneration all four times that it was tested.

Most of the stems remained alive in the inhibiting solution and regenerated when returned to fresh sea water.

The inhibiting action of the colony water must be caused by something released to the water from the tissues, because the water loses its inhibiting power when heated. The inhibitor is thermally unstable. Temperatures of 90°–100° for a few minutes destroy it. The inhibitor is not a gas, as seen from the fact that it was collected in water through which air was bubbling vigorously.

TABLE 5

RELATIONSHIP BETWEEN CONCENTRATION OF INHIBITOR AND DEGREE OF INHIBITION

Hours Inhibitor in Contact with Colony	Number of Stems Tested	Number Completely Inhibited	Percentage Completely Inhibited	Hours' Delay of Stems Regenerating
1	40	10	25	5
2	40	26	65	8
3 5	50	43	86	13
12	40	40	100	......

Cut colonies were not the only source of inhibitor found. Adult hydranths are excellent sources. Ten living hydranths in 50 cc. of sea water with 10 cut stems almost completely inhibited the regeneration of the stems. Regenerating stems themselves inhibit one another. In 16 experiments, the average regeneration rate of 10 stems in 50 cc. of standing sea water was found to be faster than that of 20 or 30 stems in the same volume of standing sea water. The average regeneration time of the 10-stem groups was shorter by from 1 to 8 hours. It appears that both hydranth and stem tissues produce a regeneration-inhibiting substance. Some of the inhibition of stems upon one another in still sea water may be due to substances other than the nonvolatile inhibitor. This can be seen from the fact that the differences in regeneration rates between 10 and 20 stems in bubbling sea water are much less than in still sea water. In 4 experiments the differences between 10- and 20-stem groups in bubbling sea water vary from 0 to 3 hours slower for the larger group. The differences ranged from 1 to 8 hours in the standing water experiment.

It has been shown that substances produced and released by the tissues inhibit regeneration. If the inhibitor is released more readily through a cut surface, free of perisarc, than through an intact surface, then ligatured stems should not inhibit other stems as much as do unligatured stems. An experiment to determine this was conducted in the following way. Stems similar in size and appearance were cut from a single colony.

They were cut to a length of 10–12 mm. Ten were selected at random and placed in a 4-inch finger bowl containing 50 cc. of sea water. Another finger bowl received 20 stems. In a third were placed 10 stems plus 10 ligatured at both ends. The experiment was performed nine times. Each time the average rate of development in the 20-stem dish was slower than that in the 10-stem dish. Furthermore, the average rate of regeneration of the 10 stems housed with 10 ligatured stems was, in all cases but one, intermediate between the regeneration rates of the 10- and 20-stem groups. The data are summarized in Table 6. The one exceptional case, No. 7, may be disregarded, because 4 of the 10 stems did not regenerate at all, and the others regenerated slowly. This is considered evidence that a toxic agent was present in the dish, and therefore this one exceptional case is dropped from consideration. In the 8 experiments, it was found that 10 stems were inhibited by 10 ligatured stems but not as much as they were by 10 stems with open ends. The data seem reliable. The expectation on a purely chance basis for the rate of 10 stems housed with 10 ligatured stems to be less eight times in a row than that for 10 stems housed with 10 open ones is $(\frac{1}{2})^8$.

TABLE 6

A COMPARISON OF THE STAGES IN REGENERATION OF STEMS INHIBITED BY LIGATURED AND NONLIGATURED STEMS*

Expt No.	10 Stems	10 Stems Plus 10 Ligatured Stems	20 Stems
1	50 4†	44 3	42 4
2	35 5†	29 4	21 2
3	21 0†	20 1†	17 2
4	43 7†	40 1†	37 1
5	33 0†	32 2†	29 0
6	46 2	44 3	42 3
7	34 8	17 0	28.7
8	33 9†	29 4	27 9
9	42 0	36 4	35 1

* The morphological stages observed are given the hour-values for the same stages in stems which regenerated at 15° C

† Indicates average for two groups of stems from same colony done at same time.

In the first section of the paper it was demonstrated that stems start to regenerate without an open surface, provided that sufficient oxygen is present, but that they do not continue to regenerate if there is no surface open to sea water. It is now believed that sufficient inhibitor accumulates to prevent regeneration when there is no open surface.

Regeneration-inhibiting substances are produced by the tissues. They are released in sufficient quantity through open ends to permit regeneration. The concentration of the inhibitor must be lower at open ends than elsewhere. Therefore one reason for regeneration close to a cut surface is the relatively low concentration of inhibitor there.

DISCUSSION

The following is a review and discussion of the events leading to the regeneration of a hydranth and the determination of the locus of regeneration.

The first step, and a necessary one, is the isolation of the stem from its hydranth (Child, 1929). Under favorable conditions no extrinsic stimuli are necessary for regeneration. Regeneration commences in isolated stems although the stems are ligatured and the extrinsic factors are unchanged. A cut is unnecessary, as seen from the observation that regeneration can occur in nature during the winter in the stems of intact *Tubularia* without any cut. Possibly the old hydranth must be removed, because, when present, it prevents regeneration by removing from the circulation the raw materials used in hydranth formation. Support for this belief comes from experiments on other Hydrozoa. When a head and upper body of a *Hydra* were grafted onto the body of another *Hydra* (King, 1901), the graft appeared, in the course of days, to move slowly down the body of the host until it reached the base, where it separated from the host. Such apparent motion by the graft through the host tissues might result from continual absorption of more basal regions and faster growth in the more apical regions. The vital staining experiments of Kanajew (1930) indicate that, during the growth of the head in *Hydra*, growth proceeds by the addition of cells which were formerly body cells. Goetsch (1929) gives evidence that, during regeneration, cells from other regions migrate into regenerating regions. These facts show that the head region removes materials from more basal regions during growth and during regeneration. It is thought that an adult hydranth removes these materials rapidly enough to inhibit regions which might regenerate if they had the raw materials.

Several factors work together in determining the locus of regeneration. Distal regions have an inherent advantage over more proximal regions. Other factors promoting regeneration locally are a relatively high local oxygen supply and a relatively low local inhibitor concentration. When both distal and proximal ends are open, both ends have relatively high oxygen supply and relatively low inhibitor concentration. The inherent gradient structure in that case determines the place of regeneration. When a narrow-mouthed cap with oxygen is placed over a distal end and left there, the distal region is favored by its gradient structure and the high oxygen, but inhibiting substances prevent the regeneration there. When a large-mouthed cap with oxygen is placed over a distal end, the inhibitor concentration is slightly greater in the distal region but the high oxygen and gradient determine the regenerant there. If, as in the last case, a large-mouthed cap, but without oxygen, is applied distally, the balance is swung the other way by the difference in oxygen concentration. Thus, all three—gradient structure, inhibitor, and oxygen—are shown to play a part in determining the locus of regeneration.

There may be a common mode of action of all these factors which influence the locus of regeneration. All may alter metabolic rate locally and consequently change the rate of raw material removal from the coelenteron locally. Evidence presented by Child and his associates (see reviews by Child, 1929, and by Barth, 1940*b*) indicates that the region in which a regenerant forms metabolizes more rapidly than do other regions. Distal regions have higher rates of oxygen consumption than proximal regions (Hyman, 1926; Barth, 1940*a*). High local oxygen supply at a cut end would increase the metabolic rate, since oxygen consumption increases with increased oxygen tension (Barth, 1940*c*). Whether the inhibitor also affects the metabolic rate is not yet known. It was suggested by Miller (1939) that oxygen and high temperature which favor regeneration locally do so by increasing the metabolic rate. An increase in the metabolic rate would presumably be followed by a more rapid extraction from the coelenteron of the raw materials of regeneration.

SUMMARY

1. Removal of dominating regions is the stimulus to regeneration.

2. Regeneration can commence without the stimulus of an open surface.

3. It has been demonstrated that an inhibitor to regeneration is produced by the tissues of *Tubularia.*

4. A cut surface is necessary during regeneration because through it pass oxygen and regeneration-inhibiting substances.

5. The locus of regeneration is determined by the interaction of three factors: the stem level, the oxygen concentration, and the inhibitor concentration.

6. Supporting evidence for the circulation theory of dominance is presented.

LITERATURE CITED

Barth, L. G. 1938*a*. Oxygen as a controlling factor in the regeneration of *Tubularia.* Physiol. Zoöl., **11**:179.

———. 1938*b*. Quantitative studies of the factors governing the rate of regeneration in *Tubularia.* Biol. Bull., **74**:155.

———. 1940*a*. The relation between oxygen consumption and rate of regeneration. *Ibid.*, **78**:366.

———. 1940*b*. The process of regeneration in hydroids. Biol. Rev., **15**:405

———. 1940*c*. The role of oxygen in regeneration of *Tubularia.* Biol. Bull., **79**:360.

Child, C. M. 1929. Physiological dominance and physiological isolation in development and reconstitution. Arch. f. Entw'mech., **117**:21.

Driesch, H. 1899. Studien über das Regulations-vermögen der Organismen. II. Arch. f. Entw'mech., **9**:103.

Goetsch, W. 1929. Das Regenerationsmaterial und seine experimentelle Beeinflussung. Arch. f. Entw'mech., **117**:211.

Hyman, L. H. 1926. The axial gradients in Hydrozoa. VIII. Biol. Bull., **50**:406.

Kanajew, J. 1930. Zur Frage der Bedeutung der interstitiellen Zellen bei *Hydra.* Arch. f. Entw'mech., **122**:736.

King, H. D. 1901. Observations and experiments on regeneration in *Hydra viridis.* Arch. f. Entw'mech. **13**:135.

Loeb, J. 1892. Untersuchungen zur physiologischen Morphologie. II. Würzburg.

Miller, J. A. 1937. Some effects of oxygen on the polarity of *Tubularia crocea.* Biol. Bull., **73**:369.

———. 1939. Studies on polarity in *Tubularia* regenerates. Anat. Rec., Suppl., **75**:38.

Morgan, T. H., and Stevens, N. M. 1904. Experiments on polarity in *Tubularia.* Jour. Exper. Zoöl., **1**:559.

Peebles, F. 1931. Some growth-regulating factors in *Tubularia.* Physiol. Zoöl., **4**:1.

Torrey, H. B. 1912. Oxygen and polarity in *Tubularia.* Univ. of Calif. Publ. Zoöl., **9**:249.

Zwilling, E. 1939. The effect of the removal of perisarc on regeneration in *Tubularia crocea.* Biol. Bull., **76**:90.

STUDIES ON HOMOPLASTIC AND HETEROPLASTIC TRANSPLANTATION OF METANEPHRIC PRIMORDIA TO THE COELOM OF THE CHICK EMBRYO

(Two Plates)

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THE many diverse experiments in which animal tissues have been transplanted have demonstrated that most adult vertebrate hosts are refractory to hetero-plastic tissues from adult vertebrate donors. In contrast, tissues transplanted between early vertebrate embryos have usually lived and differentiated. This dissimilar behavior of embryonic and adult hosts makes it apparent that species specificity has its origin sometime during development.

Several experiments in which transplants have been placed on the chorio-allantoic membrane of the chick embryo have provided evidence of the time of origin of specificity in this form. Among the earliest of these investigations was that of Murphy (1913). He found that embryonic rat tissue grew upon the membrane for periods up to 11 days, but that implants of the same tissue to the just-hatched chick degenerated within 3 days. From these results Murphy concluded that the qualities which make the adult chicken an unsuitable host for heteroplastic tissues develop during the last 2 days of intrashell life. In further experiments Murphy (1914) inoculated the chorio-allantoic membranes of 12- and 15-day chicks with macerated rat sarcoma. The resultant grafts were removed at intervals beginning on the seventeenth day of host incubation. Active growth of the engrafted tissue continued through the eighteenth day but ceased on the nineteenth day, when connective tissue began to penetrate the grafts at their peripheries. Necrotic centers developed by the twentieth day, at which time polymorphonuclear leucocytes and round cells began to enter the grafts. At this stage connective-tissue capsules had formed around the grafts, and strands had begun to penetrate the central regions. The preceding observations were taken as evidence that the specificity of the host appeared suddenly rather than gradually, and the statement was made (p. 521) that "the chick at about the time of hatching develops a defensive mechanism against the tissue of foreign species." Subsequent experiments gave the same results, and Murphy (1926, p. 15) again observed that ". . . . the avian embryo has no defensive mechanism against the growth of heteroplastic tissues till after the eighteenth day of incubation, but after this period it develops rapidly and attains its full power at or about the time of hatching."

The experiments of Murphy have thus established the hatching period as the definitive time during which the chick becomes reactive. A recent statement by Oakley (1938) demonstrated that Murphy's conclusions have found acceptance. On the basis of the results of Murphy (1913), Oakley stated (p. 115) that ". . . . the chick's individuality has developed at hatching to a degree rendering impossible the development of even embryonic tissue of the same species." However, the grafts which provided most of the evidence upon which Murphy's conclusions were based were removed from the chorio-allantoic membranes of their hosts in the interval between the seventeenth day of host development and the hatching period. It is a familiar fact that during this interval the blood supply to the membrane diminishes steadily and its value as a functional adjunct decreases accordingly. Since the period during which the chorio-allantoic membrane provides optimum vascular conditions to engrafted tissues ends before hatching, transplants intended to serve as indicators of specificity origin should be established within the body tissues of the host during the definitive period in question. In the present experiments homoplastic and heteroplastic embryonic metanephric tissue was transplanted to the coelomic cavities of embryonic chick hosts and removed at different stages of development up to and through hatching.

The present report offers results which bear chiefly upon the problem of the origin of specificity in the chick embryo and the relation of specificity origin to reactive tendencies in the host. Other factors considered are (*a*) the ability of incorporated and functional tissues to survive the period indicated to be that during which species specificity is assumed to arise in the chick embryo and (*b*) its corollary, the possible relation of the lack of active functioning of transplants to their degeneration.

MATERIAL AND METHODS

White Leghorn chick embryos were used as recipients throughout and served as donors in the homoplastic experiments. Heteroplastic donor tissues were obtained from White Pekin duck embryos.

Transplantation of donor rudiments to the coelomic cavities of chick embryos was first reported by Joy (1934). This investigator placed eye rudiments from 36-hour embryos into the coeloms of 48-hour chick hosts. Later, Hamburger (1938) described a method by which limb primordia were introduced into the coeloms of 48-hour chick hosts through a slit in the somatopleure close to the lateral edges of somites in the region of operation. Eastlick (1939*a*, *b*, and *c*) employed this technique, using 60- and 70-hour hosts. In the experiments reported here, embryos of 72–80 hours' incubation were selected as hosts. In beginning an operation a small square window was made in the shell. This window was lifted to expose the embryo, following which a slit was made in the vitelline membrane and the forming chorion along a line parallel to and just beside the right body wall. The tissue to be implanted was pushed into the coelom through the slit thus made and usually came to lie above the omphalo-mesenteric vessels.

The embryos were operated under a dissecting microscope providing a magnification of 43.2 times, using intense light. Glare from the surface of the embryo was controlled by filtering light from the illuminator through a sheet of Polaroid. Also disks of this material were fitted upon the diaphragms of both oculars of the microscope. Glare was thus eliminated, and the light intensity was modified by rotation of the oculars. Donor tissues were dissected in sterile 0.9 per cent NaCl, on a warm stage. They were freed of the connective tissue by which they were bound to the Wolffian bodies, and all associated tissues were removed. In the homoplastic experiments, $\frac{1}{2}$ metanephros was implanted at each operation. Since the donor tissues in the heteroplastic operations were somewhat older, smaller fractions of the whole gland served as transplants.

The grafts obtained were fixed in Zenker-formol, sectioned at 6 and 8μ and stained in either haematoxylin-eosin, haemalum-eosin, or haematoxylin-azure-eosin. Normal stages of metanephroi were used as bases for comparison with the grafts obtained.

OBSERVATIONS AND RESULTS

Observations have been made on three series each of homoplastic and heteroplastic transplants. The series comprising each group differed by the increasing time intervals between operation and graft removal. The time relations of the hosts, donors, and grafts of these series are set forth in Table 1.

Most of the grafts recovered had become incorporated on or within the mesenteries, since in each operation the donor rudiment was introduced into the coelom at a point near the omphalo-mesenteric vessels. As a result, grafts removed from the mesenteries were most often established near these vessels and received branches from them. A few transplants became attached to the developing liver, mesonephroi, body wall, and mesenteries *in the manner described by Joy (1934, 1939).* Blood supply and connective-tissue *incorporation developed regardless of the locus of implantation.*

TABLE 1

TIME RELATIONS OF HOSTS, DONORS, AND GRAFTS

	Group I Homoplastic			Group II Heteroplastic		
Series	1	2	3	4	5	6
Donor age (in days)	7	7	7	10	14	14
Time on host (in days)	5	11	16	5	15	17–18
Age at removal (in days) Host	8	14	19	8	18	20–21
Age at removal (in days) Donor	12	18	23	15	29	31–32
Number of operations	82	73	41	14	22	49
Number grafts recovered	27	16	6	8	7	4
Number grafts studied	27	15	5	8	7	4

THE HOMOPLASTIC GROUP

Tissue incorporation in each homoplastic series was rapid. Well-organized stroma, probably of host derivation, was found in grafts of Series 1, which remained on the host but 5 days. This stroma was especially abundant in areas surrounding renal parenchyma and in the centers of the grafts. Arterioles and venous pathways ramified through the grafts, providing the stroma and renal parenchyma with an adequate blood supply (Pl. I, Fig. 1). These conditions of incorporation were found throughout the six series.

Ser. 1.—The kidney tissue in grafts of this series was equivalent in age to that of a 12-day chick embryo. The development which had occurred gave ample evidence of tissue maintenance and growth. Arborizations of kidney ducts had formed renal trees, and in a few such trees the lumina of collecting ducts were continuous with those of metanephric spheres. An occasional sphere had developed into a capsule with distinct visceral and parietal layers, and, although the majority of these capsules were without vascular supply, a few glomeruli were observed.

Proximal convoluted tubules had developed in small numbers but were not of general occurrence. Their normal differentiation was indicated by the presence of brush borders and striae in the tubule cells. Cytoplasmic vacuoles were disposed in tangential strata in the distal thirds of some of the cells. The cells of the proximal tubules had developed a pink staining reaction which, coupled with the fact that chick kidney tissue 12 days of age is normally functional (Gersh, 1937), suggested that excretory activity had begun in these tubules.

A stainable precipitate, which apparently resulted from the fixation of a viscous fluid, was found in the lumina of proximal convoluted tubules, collector ducts, and large ureteric branches. This precipitate stained pink in the lumina of the proximal tubules, while a blue reaction was typical in the larger ducts. Occasional phagocytes were present in the lumina of the larger collector ducts.

The ureters of the donor metanephroi were cut through in preparing implants. The ends of the ureters closed after transplantation, and the nephric systems which developed from the rudiments failed to communicate with the coelomic cavities of the hosts.

The stroma of the grafts was loosely organized in the central areas and was present in greater amounts than a supportive function would require. Peripherally, the connective tissue of the grafts was dense, and the grafts were inclosed by a capsule a few cells thick. In well-organized grafts, connective-tissue trabeculae radiated from the graft centers to afford support and convey blood channels to the parenchyma. Malpighian bodies were distributed peripherally in grafts of this type, and the closed ureteric stumps occupied positions corresponding to kidney hila.

In the loose stroma surrounding venous sinusoids, many connective-tissue cells were observed which were in the initial stages of hemoblast formation. In later stages of differentiation, fibrillae had disappeared, and pseudopodia had formed (Pl. II, Fig. 9). The resultant hemoblasts were the apparent source of granuloblasts and granulocytes associated with them in the connective tissue around venous sinusoids (Pl. II, Fig. 10).

Large cells with yellow-appearing cytoplasm were present in almost every graft but occupied no specific position in any graft. Their nuclei were usually basophilic, although some were yellow. While these cells occurred in grafts throughout the series and their appearance was the same in every graft, they did not appear to be renal cells and were never components of organized structures.

Ser. 2.—These grafts, removed from 14-day embryos on the eleventh postoperative day, provided donor tissue 18 days old. Growth of the parenchyma had occurred, but there was no discernible differentiation of either ascending or descending loops of Henle or distal convoluted tubules. Malpighian bodies, normal in form and having glomeruli, had in a few cases developed lumina within which a precipitate was observed. The proximal tubules were longer and of greater diameter than those of the preceding series. The cells of these tubules were acidophilic, possessed brush borders and vacuoles, and displayed cell striation comparable to the normal. Residue found in the lumina also stainde pink. Collector ducts and ureter branches were larger than those of the younger grafts but were unchanged in essential structure. Occasional phagocytes were present in their lumina. The general development of the renal parenchyma of these grafts lagged behind that of normal metanephroi of corresponding age.

The connective-tissue organization in grafts of this series was more complete than that found in grafts of the preceding series. Capsular stroma was heavier, being eight or ten cells thick in representative cases. In the graft centers, loose connective tissue surrounding venous sinusoids contained many hemoblasts as was the case in the younger transplants. However, granulopoiesis was more intense, and the proportion of granulocytes to hemoblasts had increased.

Six of the fifteen grafts examined were necrotic and displayed the following phenomena: (*a*) kidney tubules had lost their characteristic organization and had assumed the form of solid cylinders; (*b*) no Malpighian bodies were identified; and (*c*) cells of the nephric tissues were vacuolated and deep staining, and some sent ramifying processes to the connective tissue surrounding them. Many hemoblasts, round cells, and connective-tissue wandering cells were present, and a scarcity of supportive cells indicated that connective-tissue cells had been expended toward the production of blood elements.

Ser. 3.—Twenty-three-day metanephric tissue was obtained in these transplants, which were removed from 19-day embryos 16 days following implantation. The renal elements of the grafts presented no striking differentiation beyond the condition of these structures in the transplants just described.

Although most of the proximal convoluted tubules appeared normal, some of them displayed a modified histology. In the latter, cells were observed which contained cytoplasmic vacuoles varying in size from the normal small ones to enormous intracellular spaces which surrounded cytoplasm-enveloped, pyknotic nuclei. In several instances cells apparently had burst and ejected nuclei and cell debris into the lumina of the proximal tubules.

Connective-tissue establishment was different from that heretofore described only in being slightly advanced in organization. Hemopoiesis occurred in positions in which there was much vascular tissue.

THE HETEROPLASTIC GROUP

The connective-tissue organization, vascular supply, and general nature of incorporation of grafts comprising this group corresponded with those of the homoplastic transplants.

Ser. 4.—Implants of duck metanephric tissue from 10-day embryos remained on chick embryo hosts for 5 days. Kidney capsules had developed in the 15-day tissue thus obtained, and some of these were supplied with glomeruli. As in the homotransplants, cells of the proximal tubules were acidophilic, and some contained vacuoles. The remaining nephric elements commonly present in the homoplastic grafts were represented and appeared to have developed in an orderly fashion. Hemopoiesis occurred in the same positions as in the homotransplants. Hemoblasts were more numerous than granuloblasts and granulocytes.

Ser. 5.—These grafts, formed of 29-day-old duck metanephros were removed on the fifteenth day after operation. The recovered transplants presented evidence of a greatly modified renal structure. In many cases the closed ureters were expanded and appeared as extensive vesicles. This dilatation (Pl. I, Fig. 2) frequently extended through the metanephric tree to the cavities of the Malpighian bodies. Correlative with this general distention, a degeneration of some of the proximal tubules had occurred (Pl. I, Fig. 3). This degeneration, characterized by the vacuolation and rupture of cells, was identical in nature with that observed in the oldest grafts of the homoplastic group.

Notwithstanding the generally abnormal nature of the renal parenchyma, a large number of normal Malpighian bodies and collecting tubules were present. Normal proximal tubules varied among themselves in staining intensity but in no other particular. The commonplace nature of cells of these tubules was indicated by the presence in them of brush borders, striae, and vacuoles which were occasionally disposed in strata.

Hemopoiesis was intense, and granulocytes were the most numerous of the types of blood cells observed.

Ser. 6.—Duck metanephros from donors of 14 days' incubation was allowed to remain engrafted until fixation just after the hatching of the hosts. In spite of the advanced age of the graft tissue, renal structures, when normal, were entirely representative (Pl. I, Fig. 4; Pl. II, Fig. 8). Many of the Malpighian bodies were normal and vascular, but a large number of capsules contained cavities within which detritus and nuclei were found. The majority of proximal tubules were abnormal and displayed ruptured cells and lumina which contained nuclei and debris (Pl. I, Fig. 5; Pl. II, Fig. 7). The proximal ends of some tubule cells remained adherent to their basement membranes, while the remaining portions of the cells were discharged into the duct cavities. Some of the tubules were represented by a basement membrane invested by a thin layer of connective tissue. The persistence of a space bounded only by basement membrane and its investing stroma and of greater diameter than the normal proximal tubule indicated that fluid pressure maintained the turgor of these degenerate tubules. Nuclei and cell parts, associated with a precipitated substance, were present in the lumina of some of the degenerate proximal tubules. The precipitate was in small, stellate clumps, from which thin processes extended to the basement membranes of the tubules. A few leucocytes were associated with these clumps, but there was no apparent reason for their presence.

Lumina of the smaller collecting ducts sometimes contained free nuclei and cell fragments. The large ureteric branches were distended, and it is probable that fluid contained within these branches caused an observed flattening of the normal columnar epithelium. The ureter and its large tributaries were incased in a thick tunic of connective tissue, indicating response to a necessity for support created by internal pressure.

GENERAL CONSIDERATIONS

Examination of the grafts comprising all the six series has brought to attention certain features which were observed throughout the results. One of these features was the regular growth exhibited by the transplanted metanephric rudiments. In grafts of corresponding ages kidney development was identical with that reported by Atterbury (1923) for homoplastic transplants to the chorio-allantoic membrane of the chick embryo. In the older grafts of her experiments Atterbury observed that the ascending and descending loops of Henle failed to develop completely. Such a failure was characteristic of the grafts herein reported.

Another phenomenon of general occurrence was hemopoiesis. The cells of the loose connective tissue surrounding vascular channels gave origin to hemoblasts which were observed in the stages of their transformation (Pl. II, Fig. 9). The further differentiation of these hemoblasts was observed to vary with environmental conditions. If by amoeboid activity these cells traversed the walls of sinusoids to enter the blood stream, further differentiation was toward the production of erythrocytes. The ones which remained outside the lumina of blood vessels gave rise to granuloblasts and granulocytes, which remained in the vicinity of their formation to form large aggregations (Pl. II, Fig. 6). Much of the connective tissue present in the earliest grafts must have been supplied by the hosts, judging from the volume in which it was present. It can thus be assumed that host cells have reacted in accordance with their relation to the vascular channels of the grafts. This environmental control of hemoblast differentiation corresponds to the description of chick hemopoiesis given by Danchakoff (1916) and Atterbury (1923) and is presented here as further evidence of the potentialities of embryonic connective-tissue cells.

The origin of the precipitate observed in all segments of the nephric systems is undetermined. Judging from its volume, it could not have resulted from developmental processes. Since the volume increased with the ages of the grafts, it would be reasonable to assume that the precipitate represents a kidney secretion coagulated at fixation. Although strongly indicated, this origin can only be assumed on the basis of present evidence.

Further indirect evidence of the active functioning of engrafted renal tissues is supplied by the breakdown of cells of the proximal tubules. Since the ureters of the engrafted rudiments closed shortly after implantation, the introduction of a fluid into the nephric lumina apparently resulted in general distention of the kidney system. Such distention was observed in grafts of the older series, and, in Series 3, 5, and 6, proximal tubules were observed which contained vacuolated and ruptured cells. The observations indicate that small normal cell vacuoles, prevented from discharge into the proximal tubule lumina by internal pressure, coalesced to form large vacuoles. Appearances indicate that these, in turn, fused to form a vacuole surrounding the nuclei and their immediate cytoplasm. Further expansion apparently caused cell rupture and the discharge of the cell nuclei into the lumina of the tubules. Because the rupture of these cells occurred largely at the apical ends, the basal membranes of the proximal tubules, along with their investing sheaths, appeared to prevent rupture at the proximal ends of the cells. Secretion against an internal pressure could plausibly have resulted in the degeneration of proximal tubules. The active functioning of the kidney elements which developed in the grafts has therefore been strongly indicated by (*a*) the nature and staining reactions of the precipitate contained within the lumina of the proximal convoluted tubules and (*b*) the dilatation of the nephric systems, accompanied by cytological changes in cells of the proximal tubules which were apparently related to this distention in a direct way.

The peculiar yellow cells gave but one indication of their origin. In a graft of Series 4, a group of metanephric-sphere cells was observed which had neither a cavity nor continuity with the collector system. Although some of these cells had fibrillae and their nuclei and nucleoli closely resembled those of connective-tissue cells, they were much larger than the ordinary supporting cells. These facts suggested that after failure of a metanephric sphere to become established as a kidney unit its cells began to degenerate and appeared in the grafts as moribund elements (Pl. II, Fig. 6).

DISCUSSION

The most significant result of these experiments has been that the hosts did not react in any discernible way against either the homoplastic or the heteroplastic grafts. Connective tissue and blood elements, relied upon as criteria of host antagonism, gave no indication that reaction against the transplants had occurred. It is important in this connection that the aggregates of granulocytes observed arose from stem cells within the grafts themselves. Furthermore, those kidney structures which were present in grafts of all the series had grown without inhibition by the hosts. While there were degenerative changes in some proximal tubules, these found reasonable explanation when viewed as results of the functioning of the secretory elements of the grafts. In short, no degenerative changes were observed which could definitely be ascribed to host antagonism.

It will be recalled that Murphy (1913, 1914, 1926), on the basis of results obtained from heteroplastic transplantations, concluded that the expression of species specificity by the chick embryo began just before hatching and increased to a potency comparable with that of the adult during the hatching period. The heteroplastic transplants observed in the present experiments failed to give any evidence of such an abrupt and unqualified assumption of species specificity.

In the absence of the clear evidence which host reaction to heteroplastic tissues would have provided, it is necessary to consider whether the implanted tissues became adapted to their hosts or whether reactive tendencies had not developed in the hosts during the period in question. There is much to suggest that the former interpretation is the more acceptable. However, if adaptation of tissues to their hosts is assumed to allow graft persistence, the correlative assumption is that the hosts were capable of reacting. The results of Sandstrom (1934) indicate that this is the case. He found that embryonic duck metanephros from donors of ascending ages was reacted against by the chorio-allantoic membranes of chick hosts shortly after implantation, and to degrees which varied directly with the ages of the implanted tissues. On the basis of this evidence it can be assumed that heteroplastic tissues in the present experiments persisted through hatching because they became adapted to their hosts.

This conjectured adaptability may be explained by factors other than the specificity relations of hosts and donor tissues. For example, the results of many experiments have led to the suggestion that transplants of glandular tissues can survive if they continue their functions in the engrafted condition. The investigations of Kiyono and Sueyasu (1917) are pertinent in this regard. They transplanted chick, duck, turkey, rat, mouse, guinea pig, and rabbit tissues to the chorio-allantoic membranes of chick and duck hosts. The connective tissue, cartilage, and skin components of the grafts persisted longer than did glandular tissues. In this same connection Murphy (1926), studying grafts resulting from the inoculation of hashed chick embryos to the chorio-allantoic membrane, observed that the epithelial elements of the grafts diminished but that bone, cartilage, and connective tissue survived in the transplants. Sandstrom and Kauer (1933) advanced further evidence of the ability of cartilage to survive engrafting. They transplanted cartilage from 20-day duck embryos to the chorio-allantoic membrane of the chick, and, although the resultant grafts were maintained for 27 days by serial transplantation, they presented no evidence of host antagonism. In contrast with the success of passive sustentative tissues as transplants, C. J. Sandstrom (1934) advanced inability to continue functioning to explain the degeneration which he observed in engrafted duck metanephros. Similarly, R. H. Sandstrom (1934) ascribed the eventual failure of pancreatic and liver rudiments on the chorio-allantoic membrane to the same cause. The foregoing evidence indicates that active functioning by engrafted tissues prolongs their survival as transplants.

It has been previously pointed out that the oldest heteroplastic grafts in the present experiments were, judging from all appearances, functional when removed. The question now evolves whether adaptation of a donor tissue to a host, which would explain its survival, consists in the mere establishment and maintenance of such tissue in an environment which allows function. In the present case, assuming that either the donor tissue or the hosts or both were species specific at the time of graft removal, the results obtained present indirect evidence that functional transplants can override those factors which would, in the absence of complete incorporation and tissue function, act to remove them. The nature of the modifications involved and their effects upon hosts and implanted tissues remain obscure.

The alternative explanation of the observed absence of host reactivity rests in the possibility that the host either does not develop a species specificity during the period examined or this specificity is modified by factors not generally encountered in transplantation experiments. In the former event solution of the problem awaits the ascertaining of the actual period during which reactive capacities arise. In the latter case the work of Sandstrom (1936) may offer an explanation. Sandstrom made reciprocal chorio-allantoic transplantations of chick and duck tissues to duck and chick embryos. The duck tissues evoked but mild reactions from the chick membrane, while the chick grafts called forth pronounced reactions. It was concluded that no reciprocity of reactive tendencies exists between the forms examined. On this basis, and considering the present results, the chick may be accepted as an unreactive host to duck tissues. However, such an explanation of the lack of host reaction observed would be based on a host-donor relationship which may not be general among the higher vertebrates. Therefore, the nature of the results of transplantations of kidney tissue from embryos of other vertebrates, perhaps more distantly related, cannot be inferred from the evidence presented here.

It was previously indicated that six of the sixty-six grafts examined were necrotic. It is not believed that this necrosis was due to host reaction, since grafts of identical host-donor relations, allowed to remain engrafted for longer periods, failed to be attacked. In the absence of the specificity factor the results of Rous (1910) provide a possible explanation. He implanted mouse tumor and embryonic mouse tissues homoplastically beneath the skin of adult recipients and found that some of the hosts were more resistant to embryonic mouse tissue than others. In the present case the hosts from which the necrotic tissue was removed were embryonic, as was the tissue of the transplants. It is hardly likely, therefore, that either host or donor tissue was reactive and improbable that the observed graft failure resulted from antagonism by atypically reactive individuals.

On the other hand, simpler factors may explain the degeneration observed. It is possible that the blood supply was adequate for maintenance and growth of the grafts during the first few days of their residence upon the hosts but that vascularity did not increase with the subsequent growth of the tissue. Superior relative growth of the kidney tissue may thus have resulted in the necrosis observed. Finally, the necrosis may have been due to tissue injury during transplantation. In this case the degeneration would not be an index of either the suitability of the site of transplantation or the conditions required for the survival of the tissue engrafted.

SUMMARY

1. Six series of transplantations of chick and duck metanephric rudiments were made to the coeloms of 72–80-hour chick hosts. The resulting grafts were allowed to remain in the host for periods varying from 5 days to 17 and 18 days and were removed from hosts which varied in age from 8 days to just-hatched chicks.

2. Four heteroplastic grafts which remained on host embryos through the period of hatching gave no evidence of having been reacted against. It was therefore concluded that the species specificity of the chick did not appear under the conditions of the experiment.

3. Explanations advanced for this result were based on (*a*) a possible adaptation of donor to host tissues; (*b*) a possible effective resistance of established and functional transplants to host reaction during the hatching period, (*c*) a lack of reactivity of chick

hosts to duck donor tissue, and (*d*) an absence of species specificity in the chick embryo during the period covered in the experiment.

4. The transplants presented histological evidence of (*a*) active functioning by the kidney elements of the grafts and (*b*) hemopoiesis similar to that described by Danchakoff (1916).

The writer wishes to express his sincerest appreciation of the generous direction and advice of Professor C. J. Sandstrom.

LITERATURE CITED

ATTERBURY, RUTH R. 1923. Development of the metanephric anlage of chick in allantoic grafts. Amer. Jour. Anat., 31:409–37.

DANCHAKOFF, V. 1916. The wandering cells in the loose connective tissue of the bird and their origin. Anat. Rec., 10:483–92.

EASTLICK, H. L. 1939. The role of heredity versus environment in limb bud transplants between different breeds of fowl. Science, 89:17–18.

———. 1939. The pigment forming capacity of the blastoderm of Barred Plymouth Rock embryos as shown by transplants to White Leghorn hosts. Anat. Rec., 73:64–65 (abstract).

———. 1939. The point of origin of the melanophores in chick embryos as shown by means of limb bud transplants. Jour. Exper. Zoöl., 82:131–57.

GERSH, I. 1937. The correlation of structure and function in the developing mesonephros and metanephros. Carnegie Inst. Washington Contr. to Embryology, 26:33–58.

HAMBURGER, VIKTOR. 1938. Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. Jour. Exper. Zoöl., 77:379–99.

JOY, EDWARD A. 1934. The development of intra-coelomic grafts of chick primordia, with special reference to the eye. Anat. Rec., 58:72 (abstract).

———. 1939. Intra-coelomic grafts of the eye primordium of the chick. *Ibid.*, 74:461–85.

KIYONO, K., and SUEYASU, Y. 1917. Ueber die Implantation der Embryonal-Gewebe verschiedener Tierspezies in die Hühner und Entenembryonen. Org. d. med. Gesellsch. Kyoto, 14:68–114.

MURPHY, J. B. 1913. Transplantability of tissues to the embryo of foreign species: Its bearing on questions of tissue specificity and tumor immunity. Jour. Exper. Med., 17:482–93.

———. 1914. Factors of resistance to heteroplastic tissue-grafting: Studies on tissue specificity. III. *Ibid.*, 19:513–22.

———. 1926. The lymphocyte in resistance to tissue grafting, malignant disease, and tuberculous infection. I. Heteroplastic tissue grafting and the mechanism of defense. Mono. Rockefeller Inst. Med. Res., 21:5–30.

OAKLEY, C. L. 1938. Chorio-allantoic grafts of liver. Jour. Path. and Bact., 46:109–20.

ROUS, P. 1910. An experimental comparison of transplanted tumor and a transplanted normal tissue capable of growth. Jour. Exper. Med., 12:344–66.

SANDSTROM, C. J. 1934. Additional observations on heteroplastic chorio-allantoic grafts of embryonic duck kidney tissue. Physiol. Zoöl., 7:279–303.

———. 1936. Reciprocal chorio-allantoic transplants of embryonic duck and chick kidney tissue. Biol. Bull., 70:36–49.

SANDSTROM, C. J., and KAUER, G. L. 1933. Heteroplastic transplants of duck cartilage to the chorio-allantoic membrane of the chick. Anat. Rec., 57:119–31.

SANDSTROM, RUTH HOLTON. 1934. The differentiation of hepatic and pancreatic tissues of the chick embryo in chorio-allantoic grafts. Physiol. Zoöl., 7:226–46.

PLATE I

FIG. 1.—×100. Section of graft of Series 1 to demonstrate conditions of incorporation which prevailed throughout the entire experiment. Observe: (1) relation of graft to gut (*a*), mesentery (*b*), and blood supply (*c*); (2) organized stroma; (3) typical distribution of vascular channels and loose connective tissue within the graft; and (4) well-established renal parenchyma.

FIG. 2.—×100. Section of graft from Series 5 which illustrates the extensive dilatation of the entire collector system.

FIG. 3.—×400. Section represented in Figure 2 more highly magnified. Observe the vacuolation of cells of the proximal convoluted tubules (*a*), the distention of these tubules, and the debris in the duct lumina (*b*).

FIG. 4.—×100. Graft of Series 6. Presents the generally normal appearance of those transplants allowed to remain on their hosts through the hatching period. Compare the general character of the transplant with that of Figure 2.

FIG. 5.—×400. Graft of Series 6. Gross appearance of the parenchyma of a graft which remained on the host through hatching. Precipitate and cell debris may be observed in the duct lumina. Compare with Figure 3.

PLATE II

FIG. 6.—×400. Graft of Series 5. The cluster observable in the upper center (*a*) is composed of yellow cells. The cells aggregated in the upper right (*b*) are granuloblasts and granulocytes. These elements are seen to occur in areas bounding renal parenchyma (*c*).

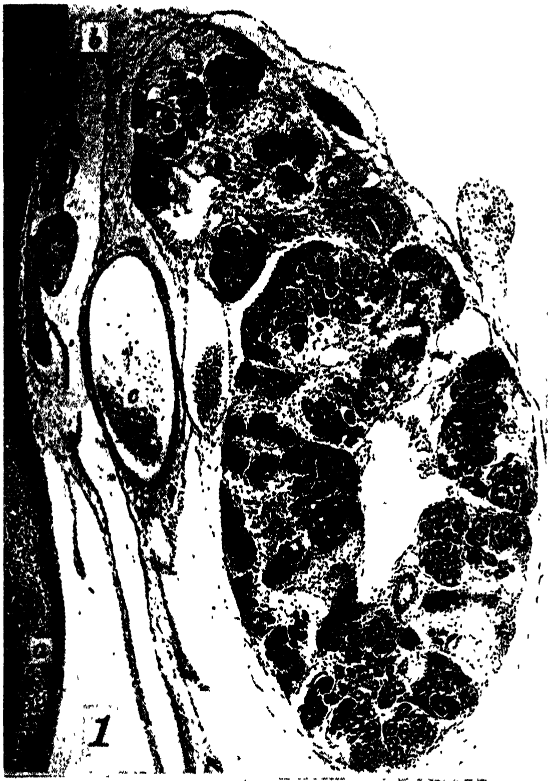
FIG. 7.—×950. Proximal convoluted tubules undergoing degeneration. From the graft represented in Figure 5.

FIG. 8.—×950. Normal proximal convoluted tubule (*a*), containing brush border. In the same field are tubules in the process of degeneration (*b*). From the graft represented in Figure 5.

FIG. 9.—×950. Amoeboid hemoblasts in the loose connective tissue of a graft of Series 4 (*a*).

FIG. 10.—×950. Granuloblasts (*a*) arising in loose stroma surrounding venous sinusoid (*b*).

PLATE I



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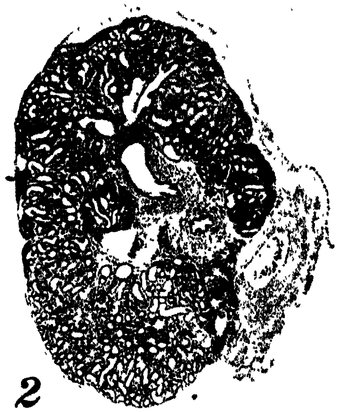
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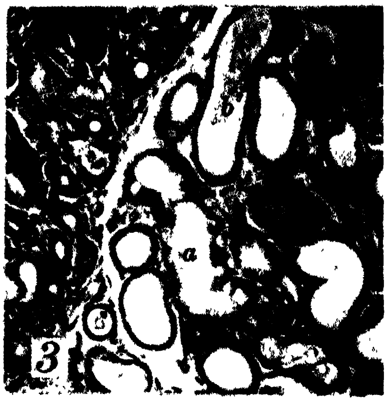

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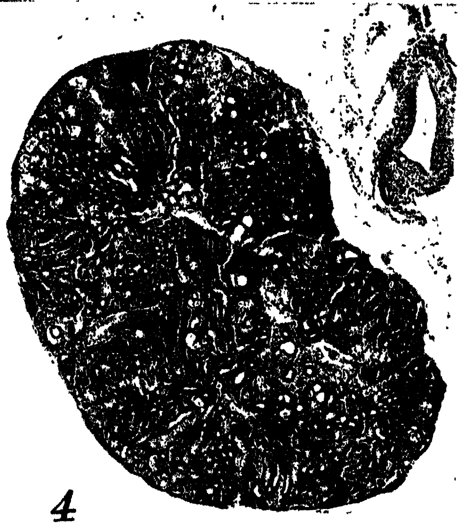
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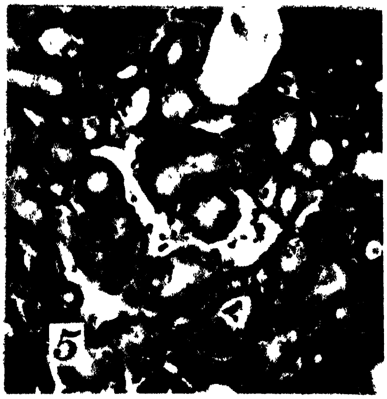
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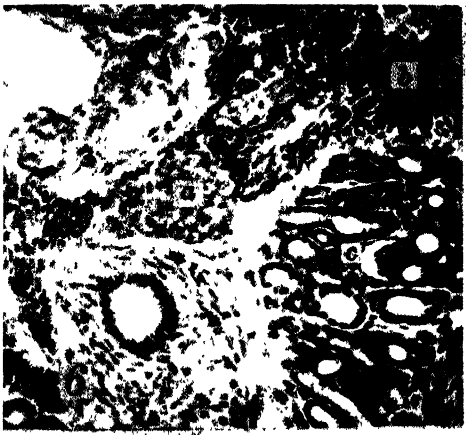
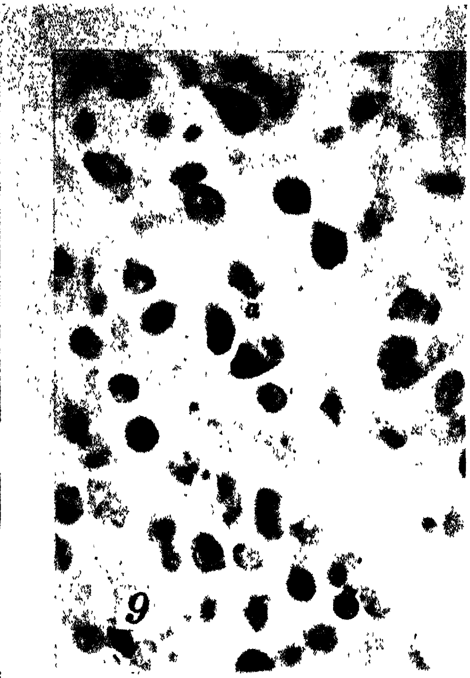
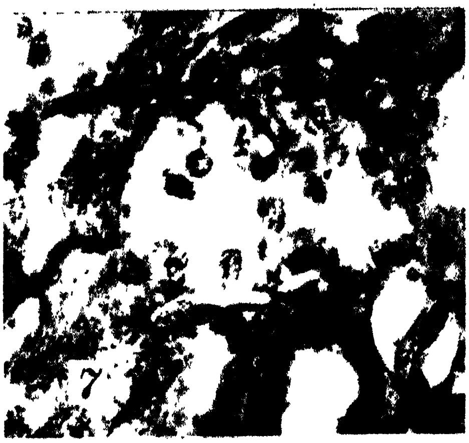
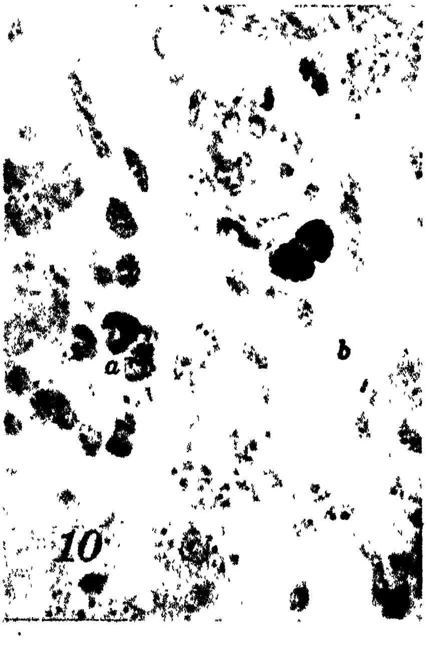
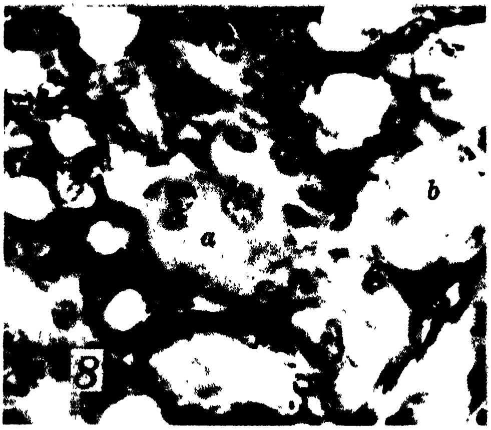
PLATE II











TRANSPLANTATION OF LIMB PRIMORDIA OF HOMOZYGOUS AND HETEROZYGOUS CHONDRODYSTROPHIC ("CREEPER") CHICK EMBRYOS[1]

(One plate)

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IN THE experiments to be presented in this paper an attempt has been made to analyze certain aspects of the action of the "Creeper" factor in the chick, using the method of embryonic transplantation. The "Creeper" material has been chosen because the effects of this hereditary factor on development have been studied to a considerable extent by Landauer and his associates (1931–40); thus a sound basis for further investigations has been laid.

Landauer and Dunn (1930) have shown that this mutation is a dominant lethal and that the heterozygous phenotype is identical with chondrodystrophy described for mammals. In the heterozygous condition (Cpcp) the "Creeper" factor is expressed mainly by a considerable shortening of the legs. The tibia is bent, the fibula is abnormally strong and nearly of the full length of the tibia, with which it is fused on both ends. In the homozygous condition (CpCp) the "Creeper" factor is usually lethal at the fourth day of incubation. At that stage the embryos are retarded considerably in growth and differentiation; the heads are disproportionately small and asymmetrical, blood pools are apparent both intra- and extra-embryonically. A small number of homozygotes survive this critical period, but they never reach the hatching stage. They are characterized by a considerable growth reduction. Wings and legs are extremely short, the latter being rotated craniad. The feet seem to be attached directly to the flank, a condition which is known as "phocomelia" and occurs as a rare congenital malformation in mammals and man. The skull and eyes show marked abnormalities. Throughout the present paper these two types of homozygous embryos will be designated as "early lethal" and "phocomelic" embryos respectively.

When early primordia of Cpcp and CpCp embryos are transplanted heterotopically onto normal embryos, their genetic milieu is altered as well as their relation to inductive and other correlative factors. Nutrition and innervation will be supplied by the host. A priori, the transplants may be expected to follow either one of two courses. They may differentiate into typical Creeper- or phoco-like structures. This would mean that the gene-controlled processes which result in the phenotypic malformations are, from the moment of operation, entirely intrinsic in the primordium and not modifiable under the conditions of the experiment. In this instance, variations of the stage of operation, of the site of transplantation, and of the genetic constitution of the host would be required for further analysis. The other alternative would be that the transplants would not exhibit the characteristic Creeper or phocomelia features; they might even be expected to develop normally in the normal milieu. The obvious conclusion would be that the primary gene action is extrinsic to the transplant and that the phenotypic expression is brought about by mediation of inductors, hormones, or other agents. Different primordia, and even partial developmental phases of the same primordia, might behave differently in this respect.

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[1] This investigation was aided by a grant from the Rockefeller Foundation. I wish to express my appreciation of the valuable assistance of Mr. Eugene L. Keefe. The results were briefly reported in Hamburger (1939*b*).

Of particular interest will be the fate of transplants taken from "early lethal" embryos. The fact that some embryos of CpCp constitution do survive and the results of tissue culture and chorio-allantoic grafting experiments by David (1936) indicate that lethal tissue is viable beyond the critical stage (the fourth day of incubation). The transplantation of such primordia into an optimal milieu should reveal their full morphogenetic and histogenetic potencies and supply additional information concerning the causes of lethality of the CpCp condition.

With these possibilities in mind we chose limb primordia as the first test objects because they are affected most drastically by the Cpcp and by the CpCp genotype and because the method of the limb-bud transplantation had been worked out previously. Fortunately, the early lethal embryos are still alive at a stage when their wing and leg primordia are easily accessible to transplantation.

MATERIAL AND METHODS

Material.—The Creeper material came from the same stock of the Storrs Agricultural Experiment Station, Storrs, Connecticut, as that which had been used by Landauer and his co-workers.[2] The material used for hosts was a pure-bred strain of White Leghorn raised in St. Louis County, Missouri.

Method of operation.—The method of limb-bud transplantation has been described previously (Hamburger, 1938, 1939*a*). Since the Cpcp embryos cannot be distinguished from normal embryos at the time of operation (third day of incubation), an attempt was made to raise the donor embryos. This necessitated a slight modification of the operative procedure. A window was made in the shell of the donor embryo. Its leg-forming area was vital stained *in situ* with neutral red, cut out by means of a glass needle, and transferred in a micropipette to the previously prepared host embryo. The windows of donor and host embryos were sealed in the usual manner and both embryos returned to the incubator.

Stages at operation.—The donor embryos ranged from 49 to 74 hours of incubation (19 to over 30 somites). The limb primordia varied between Stages 1 and 5 (Hamburger, 1939*a*, p. 349); i.e., from stages in which the thickening of the limb area was not yet visible externally to those of medium-sized buds. The limb primordia of CpCp embryos (of the same age) are usually retarded and their mesoderm less dense, so that they are less prominent than normal (Landauer, 1932). They are never larger than Stage 3 (slight swellings). The host embryos were between 64 and 72 hours of incubation (limb-bud Stages 2–4).

Site of transplantation.—The limb primordia were transplanted either to the right flank of the host embryo between the wing and leg bud or into the coelomic cavity. Both locations have been shown to be favorable for a normal differentiation of limb transplants. In general, the morphogenesis and the histological differentiation of the limb skeletons proceed normally, except for the joint formation in cases which receive inadequate nerve supply or none at all. The occurrence in the present material of ankylosis and of other deficiencies at the articulating surfaces, characteristic of noninnervated skeletons, are therefore a result of the transplantation as such and cannot be attributed to the Creeper factor (see Hamburger and Waugh, 1940).

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[2] I wish to express my deep appreciation of the co-operation of Dr. W. Landauer, of Storrs, who supplied the material and made his collection of slides available for the present investigation.

DIAGNOSIS OF THE DONOR EMBRYOS

CpCp embryos.—Early lethal embryos can usually be recognized at the beginning of the third day of incubation (Landauer, 1932; Rudnick and Hamburger, 1940). The most reliable criteria are shortness and incomplete flexure of the head; asymmetries in the head, left eye and ear primordia being smaller than right ones; blood pools occurring intra-embryonically and in the area vasculosa, particularly at the level of the leg primordia. Most of the donor embryos, selected according to these criteria, were fixed after operation and the diagnosis reaffirmed on whole mounts. Of 45 embryos diagnosed as early lethals, 23 gave good transplants, and all showed unmistakable CpCp characteristics (see Table 1). To these, 1 transplant was added which showed the same typical CpCp features but whose donor, at 71 hours of incubation, had been protocoled as "probably normal though anemic and slightly retarded." This embryo was, in all probability, a phoco-embryo which at that early state was not identifiable as such.

TABLE 1

SURVEY OF LIMB TRANSPLANTATIONS[3]

DONORS	TOTAL	HOST DIED	TRANSPLANT ABSENT OR POORLY DEVELOPED	TRANSPLANT WELL DEVELOPED	GENETIC CONSTITUTION OF POSITIVE CASES		
					Cpcp	cpcp	Doubtful
CpCp	46	14 = 31 per cent	8 = 17 per cent	24* = 52 per cent	. .	. . . . .	. . . . .
Cpcp and cpcp .	56	14 = 25 per cent	9 = 16 per cent	33 = 59 per cent	19†	10‡	4

* Seven wings, 17 legs. † Seven donors raised. ‡ Two donors raised

Identification of Cpcp and of normal (cpcp) embryos.—These two classes are indistinguishable on the third day of incubation. The direct way of establishing their genetic constitution by raising the donor embryo to an age of 11 days or older was successful in 9 cases; 7 of these were identified as Cpcp and 2 as cpcp. The transplants from the latter were normal, as expected; all 7 transplants from the Cpcp donors showed typical Creeper-like abnormalities (see Table 1). This difference between Cpcp and cpcp transplants provided a basis for the identification of those transplants whose donors had died too early. Twenty such transplants segregated clearly into two groups: 12 with Creeper-like malformations and 8 transplants with no other deficiencies than are characteristic for normal transplants (ankylosis, occasional hypodactyly, etc.). Four doubtful cases were discarded. If we consider the 12 Creeper-like transplants as of Cpcp constitution, then we obtain a ratio of 19 Cpcp : 10 cpcp.[3] The fact that this ratio is almost exactly the expected ratio 2 : 1 is considered further evidence that our diagnosis is correct.

Table 1 summarizes the results.

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[3] These figures differ from those given in a preliminary report (1939*b*). A careful re-examination of the material resulted in the elimination of several cases; a few new cases have been added.

DESCRIPTION OF THE Cpcp TRANSPLANTS

Only leg primordia were transplanted, since the wings are not markedly affected by the Cp factor. Most embryos were fixed at 11–12 days of incubation, a few at 16–17 days of incubation. Eighteen specimens were stained in methylene blue, and 1 was sectioned. For comparison the following were available: (1) Creeper embryos (donors and nonoperated specimens of corresponding stages); (2) normal embryos (hosts and nonoperated embryos); (3) a large material of leg transplants of normal onto normal embryos.

Nonoperated Cpcp legs, at 11–12 days of incubation (Fig. 1), show the following characteristics (Landauer, 1927 and 1931): All elements are considerably shorter than normal. In the more extreme variants the individual elements are more plump and stubby than normal. The femur is usually slightly bent, and, if so, hypertrophied periosteal bone is deposited in the concavity. The tibia and fibula are more severely affected than any other element. The tibia is greatly reduced in size, its diaphysis is bent in a varying degree; the concavity is completely filled with hypertrophied bone. The fibula which in normal legs is a slender rod less than one-half the length of the tibia, is stronger than normal and of the same length as the tibia. In normal legs it articulates with the proximal epiphysis of the tibia and fades out into a fibrous strand at its distal end; in Creeper legs both ends are fused with the two epiphyses of the tibia.

The four best-developed Cpcp transplants (among them two cases whose genetic constitution had been established directly by raising the donor) show exactly the same clear-cut symptoms. Case cl 84 (Figs. 1–3) may serve as an illustration. The transplant (right leg bud, Stage 4, 67 hours of incubation) had developed on the right flank of the host. Both donor and host were fixed at 11 days of incubation. The transplant (Fig. 2) is almost exactly of the same size as the left donor leg (Fig. 1), and both are approximately 25 per cent shorter than the host leg (Fig. 3). Both the donor and the transplanted leg show the characteristic curvature of the tibia, the periosteal hypertrophy at the concavity, and the abnormally long fibula which is fused with the epiphyses of the tibia. Even minor details such as the degree of cartilage erosion in the diaphyses of the long elements are identical in transplant and donor leg and slightly advanced in comparison with the host leg. (This precocity in cartilage resorption is not a constant feature of Cpcp embryos.) In another respect the transplant is intermediate: the individual elements of the donor leg show the characteristic stubbiness of the Creeper type; the transplant cartilages are more slender (cf., e.g., the phalanges). Other transplants are of the Creeper type, however, so that no generalization can be made from this observation.

All transplants except the 4 mentioned thus far show higher degrees of malformation. Two different types of deficiencies can be distinguished. Certain abnormalities represent clearly an accentuated Creeper condition, whereas others are identical with those found in transplanted normal limbs and must be considered as nonspecific effects of the transplantation per se. Both the exaggerated Creeper effect and the transplantation effect may be found in the same specimen. The following are classed as aggravations of the Creeper condition: (1) tibia and fibula fused entirely and represented by a broad, flat cartilage plate; (2) tibia and fibula separate, but shapeless and with no condyles (in one extreme case, they are missing altogether, the femur and three typical toes being present); (3) deformations of the femur, which were found in 4 cases; (4) metatarsals occasionally bent and in rare instances partly fused, as is characteristic of higher degrees of nonoperated Creeper legs. All these features, in particular the malformations of tibia and fibula, are exaggerations of what is found in Creeper legs and are in striking contrast to the abnormalities found in normal, transplanted legs. In an unselected material consisting of 40 normal leg transplants, an abnormally short tibia was found only twice. In 1 of these cases it was bent in a Creeper-like fashion. The fibula of normal transplants was frequently missing (9 cases) but never showed a tendency toward growth beyond its typical proportions. Superimposed on these Creeper effects are others which are characteristic of transplanted normal legs (Hamburger and Waugh, 1940) but which never occur in nonoperated Cpcp embryos. Most frequent among them are hypodactyly and hypophalangy and a combination of both. Hypodactyly was found in 11 of 19 Cpcp transplants, the smallest (first) toe being most frequently absent, as is the case in normal transplants. Ankylosis is common in noninnervated limbs and is definitely not a Creeper effect but due to lack of innervation.

Growth reduction.—Landauer (1934) has made an extensive study of the growth reduction of the different elements of the Cpcp legs. He found differential effects, the tibia being most excessively reduced in length. In general, he found that the growth reduction is in proportion to the final length of each element and that a proximo-distal gradient of growth retardation exists.

In order to obtain an approximate estimate of the size reduction of the Cpcp transplants, the lengths of their femora were measured and compared with the femur lengths of donors and hosts wherever they were available or with the data given by Landauer for the femora of Creeper and normal legs of corresponding stages (Landauer, 1934, Tables 3 and 4). The tibia was not chosen on account of its atypical shape. In the stages under consideration the average length of a nonoperated Cpcp femur is 20 per cent below that of a nonoperated cpcp femur. The transplanted Cpcp femora were found to be approximately 40 per cent shorter than nonoperated cpcp femora (average of 10 cases) which is a growth reduction of 20 per cent in excess of the Cpcp donors. This additional growth restriction can be accounted for readily since we have found previously that normal leg transplants rarely reach the full size of normal legs. The average growth reduction of the femur was calculated as 20 per cent (Hamburger and Waugh, 1940, Table 2). This is exactly the amount by which the nonoperated Cpcp femur exceeds the transplanted Cpcp femur. The total growth reduction of the Cpcp transplants (which is proportional to that of the femur) can be considered as the cumulative effect of the Cp factor and the transplantation per se, each contributing 20 per cent. It follows that the Cpcp transplants have retained their intrinsic limited growth rates as well as their morphogenetic characteristics.

DESCRIPTION OF THE CpCp TRANSPLANTS

As has been indicated previously, all donors except one (probably a phoco-embryo) were identified as early lethal embryos at the stage of operation. Both wing and leg primordia were transplanted. Most of the successful cases (17 legs, 7 wings) were fixed at 12 days of incubation; a few were incubated for 17 days. Four specimens were sectioned; the others were stained in methylene blue.

Table 1 shows that limb primordia taken from early lethal embryos shortly before death not only survive but yield almost as high a percentage of well-developed transplants as that of the Cpcp series. All CpCp transplants were found in a healthy condition. The viability of limb primordia is not impaired by the CpCp genotype.

The appearance of all transplants of this group is very uniform: they are extremely small and resemble closely the phocomelic limbs of those CpCp embryos which survive the critical fourth day of incubation ("phoco-embryos"). The skeleton of the latter was described by Landauer (1933, 1939) as follows: The legs are rotated 180° in their longitudinal axis and point cephalad. Their proximal elements are extremely shortened so that the foot seems to be directly attached to the body wall ("phocomelia"). The fibula is almost as large as the tibia and thus resembles the Cpcp condition. Both tibia and fibula are fused in a varying degree. The same holds for radius and ulna. In some specimens femur and humerus are present only as extremely short rudiments. Hypodactyly was found occasionally in legs. Striking abnormalities were discovered in the histological structure of the skeletal elements: calcification and periosteal ossification are completely inhibited, and the cartilage zones characteristic of the epiphyses of the long elements are absent. Our own material of nonoperated phoco-embryos stained in methylene blue showed, in addition, that the long elements are frequently flattened and atypical in shape. Their articulating surfaces are not clearly sculptured, condyles, etc. are barely indicated, yet the articular clefts are present. The abnormalities in metacarpals, metatarsals, and phalanges are even more pronounced (Figs. 4 and 6). Usually all elements of a digit or a toe are fused into a long, tapering cartilaginous rod with no indication of the typical hourglass shape of the phalanges. The joints are either completely missing or indicated by a fine slit. The boundary between metatarsals (or metacarpals) and first phalanges is usually indicated by an abrupt narrowing of the rod. The proportions of digits and toes are rather typical despite the fusions.

The condition of the best-developed CpCp transplants differs surprisingly little from this description (cf. Figs. 4 and 5; Figs. 6 and 7). The transplants show frequently an additional abnormal feature—the fusion of adjacent long elements. Such ankyloses are characteristic of nerveless transplants, as was mentioned above. However, it is doubtful whether, in the present case, the fusions can be attributed entirely to the lack of innervation, since the condition in the distal parts of nonoperated phocomelic limbs indicate that the Cp factor in a double dose has the tendency to affect skeletogenesis in the same direction. The abnormalities in the shape of the individual elements, together with the fusions and the complete absence of typical articulating surfaces, tends to eliminate the characteristics of the individual cartilages. Only their relative positions and size relations are maintained.

A number of specimens represent higher degrees of malformation, as was the case in the Cpcp transplants. Tibia and fibula were occasionally fused completely; radius and ulna remained more frequently separate, though they might be fused at their ends. Hypodactyly (sporadic in nonoperated phoco-legs and in normal leg transplants) was again quite common in legs, though less frequent than in Cpcp transplants, and usually resulted in the loss of the first (smallest) toe. All but one wing showed the complete number of digits. Again, these deficiencies must be attributed, in part, to the transplantation per se.

The 4 sectioned cases (12 and 17 days of incubation) showed all the histological characteristics found by Landauer in phoco-limbs of the same stages: absence of typical cartilage zones at the epiphyseal ends, complete absence of periosteal ossification, and only the beginning of excavation of the diaphyses. These deficiencies are the more remarkable as the older transplants are incorporated in a host in which ossification and cartilage breakdown are in advanced stages. They demonstrate the complete independence of the transplant from the host, with respect to morphogenesis and histogenesis.

Growth reduction.—Recently, Landauer (1939) has published the growth curves of the skeletal elements of phoco-embryos. These data enable us to compare the growth of early lethal transplants with that of nonoperated phoco-limbs which they resemble so closely in morphological respect. Measurements were made on 10 leg transplants, all fixed at 12 days of incubation. The measurements are necessarily crude and give only a rough estimate. The average length of the transplant femur was found to be 2.9 mm., as compared to 3.3 mm. of phoco-embryos of the same stages (=15 per cent reduction). The corresponding figures for the tibia are 2.3 and 3.3 mm. (=30 per cent reduction). This growth reduction in excess of the phoco-legs must, again, be attributed to the transplantation as such. Although only one stage was checked, it appears that the inherent growth rate of a limb bud from an early lethal CpCp embryo is of the same order of magnitude as that of a surviving CpCp leg. Another fact stands out: The tibia is more seriously affected than the femur. This is in full agreement with earlier observations on normal limb transplants, on nonoperated and transplanted Cpcp legs and on phoco-legs; all of which indicate a greater susceptibility of the tibia primordium as compared to the femur.

In summary, wing and leg transplants from early lethal CpCp embryos are viable but show the same restrictions in their morphogenetic, histogenetic, and growth potencies as were found in the surviving CpCp embryos.

DISCUSSION

VIABILITY OF PRIMORDIA FROM EARLY LETHAL EMBRYOS

The survival of the phocomelic embryos proves that CpCp structures are viable under certain conditions. David (1936) has shown the same for several tissues of early lethal embryos. He reared isolated heart, limb, and other primordia of 72-hour CpCp embryos *in vitro* and as chorio-allantoic grafts. In tissue culture they remained healthy and retained their growth activity for as long as 27 days. Chorio-allantoic grafts included well-differentiated, though not typically shaped, cartilages from limb primordia; furthermore, muscle and nervous tissue and feather follicles were present. Histological differentiation was normal except for the limb cartilages, in which the cartilage zones were absent. The same deficiency is characteristic of nonoperated phocomelic limbs and was found occasionally in chorio-allantoic grafts of limb cartilages from normal embryos. Our own material demonstrates a high degree of morphogenetic potency in addition to growth and histogenesis. Healthy legs and wings of the phocomelic type, including cartilaginous skeleton, musculature, tendons, and skin with feathers, were found on the flank or in the coelom of normal hosts of 17 days of incubation. The Creeper factor in double representation restricts the developmental potencies, but it has shown no cell-lethal or organ-lethal effect in the structures which have been transplanted so far. Similar potency tests applied to primordia which have not yet been investigated should indicate whether the immediate cause of the lethality resides in a single organ or if death is caused by deficiencies in an integrating mechanism.

EARLY DETERMINATION AND CAUSATION OF THE CREEPER CHARACTERS

Previous experiments have shown that the limb primordia of normal 48-hour chick embryos will differentiate into normal wings and legs if transplanted heterotopically.

(Certain qualifications of this statement with respect to the pigment cells, which must be supplied by the host, with respect to details of joint formation, which depend on complete innervaton, and with respect to size reduction do not concern us in the present discussion.) The present experiments show that the modification of development which is caused by the Creeper factor is likewise fixed by at least as early as the fifty-fourth hour of incubation and cannot be reversed by implantation into a genetically normal host. This holds for the Cpcp as well as for the CpCp primordia. At the time of operation the latter are thickenings or small buds. The fact that the skeleton differentiating from early lethal CpCp embryos is identical with that of phocomelic embryos is further convincing evidence that both are genotypically identical. All the morphological, histological, and growth abnormalities are faithfully reproduced in Cpcp transplants. As was to be expected the development of most transplants was impaired, in addition to the Cp effect, by the transplantation per se, partly in the form of an accentuation of the Creeper characteristics and partly in the form of hypodactyly, hypophalangy, and other deficiencies which are not among the Creeper symptoms but are characteristic of normal limb transplants.

It must be emphasized that the relative self-differentiation of the Creeper phenotype is demonstrated only for the particular conditions of the present experiments. It is possible that primordia removed at earlier stages or reared in a genetic milieu different from White Leghorn might behave differently.

The fact that the transplants were incorporated in the normal host circulation deserves special emphasis. The limb primordia were transplanted in a developmental stage at which their blood vessels were not differentiated yet. Therefore, they were supplied with normal nutritive material and normal hormones throughout their differentiation. It results that all agents carried in the blood circulation are ruled out definitely as causal factors in chondrodystrophy and phocomelia. Landauer (1929) and Fugo and Witschi (1938) have come to the same conclusion with respect to hormone deficiencies. The former author has failed to find appreciable abnormalities in the most important hormone-secreting glands of chondrodystrophic chicks, and the latter investigators have found that hypophysectomy at 33–38 hours of incubation does not result in chondrodystrophy.

NONSPECIFIC GROWTH RETARDATION AS THE PRIMARY GENE EFFECT

Landauer has shown that the structural deficiencies of Creeper embryos, as they develop, are accompanied, or even preceded, by growth reductions. He interprets the primary gene effect as a general growth retardation of the entire embryo at early stages of development, the localized phenotypic manifestations merely reflecting a differential susceptibility of the primordia at the stages when the growth-restricting agents are in operation. The morphogenetic and histogenetic abnormalities of the Creeper skeleton are considered as secondary effects. Such an interpretation implies a causal relation between growth and histogenesis. Chondrodystrophy and phocomelia are considered as expressions of differing degrees of an early growth restriction, according to the single or double representation of the Creeper factor.

If the Creeper factor is a growth inhibitor of a general, nonspecific nature, then it should be possible to simulate its effect by exposing normal limb buds to unfavorable, growth-restricting conditions. The *in vitro* experiments of Fell and Landauer (1935) were based on this reasoning. These authors reared normal leg buds in an ordinary and in a special "growth-restricting" medium. The abnormalities which they found resembled the phocomelic condition in so many structural details that the authors were inclined to assume both to be "due to agencies of the same nature" (p. 149). Similarly, David (1936) found that cartilages developing from normal leg buds on the chorio-allantoic membrane lack occasionally (but not always) the cartilage zones near the epiphyses, which deficiency, again, is characteristic of phocomelia.

By the same reasoning one would expect that a less severe growth restriction imposed on normal limb buds might result in a phenocopy of the Cpcp (Creeper) condition, likewise that a low-grade growth restriction superimposed on the Cpcp condition might result in deficiencies intermediate between chondrodystrophy and phocomelia, or perhaps in pure phocomelia. Neither one of these expectations was fulfilled in our experiments. The heterotopic transplantation of normal limb buds leads to a size reduction which averages about 20 per cent (Hamburger and Waugh, 1940) and thus provides a suitable test. This growth restriction is ascribed to an insufficient vascularization, innervation, etc., and is therefore of a general, nonspecific nature. Many of these transplants show no morphogenetic abnormalities or histogenetic deficiencies whatsoever. Others exhibit abnormalities, such as hypodactyly, etc., which are typically different from chondrodystrophy. The only deficiency which both have in common is a size reduction of the tibia (and fibula) in excess of that of other elements. This, however, is rather an argument against the thesis to be tested, since the excessive growth reduction is not accompanied by a "chondrodystrophic" bending of the tibia or a thickening, etc., of the fibula. Typical Creeper features were found in only 1 transplant among more than 100; this case was interpreted as originating from an incidentally chondrodystrophic donor (a nonhereditary form of chondrodystrophy occurs sporadically in chicks [Dunn, 1927]).

Likewise, the Cpcp transplants did not give a graded series of transitional stages between chondrodystrophy and phocomelia. It is true that the smallest Cpcp transplants had the external appearance of phocomelic limbs; but, after the skeleton had been stained with methylene blue, a clear distinction between Cpcp transplants and phocomelic limbs was possible. An unfailing criterion is the typical hourglass shape of the phalanges and the clear segregation and articulation of metacarpals, metatarsals, and phalanges in Cpcp transplants (Fig. 2) in contrast to the fusion of these elements in phocomelic wings and legs (Figs. 5 and 7). Likewise, the erosion of the diaphyseal cartilage and the periosteal ossification are characteristic of the majority of Cpcp transplants but completely missing in CpCp skeletons. These differences are maintained despite an overlap in size.

These findings seem to indicate that transplantation as such is not an adequate method of producing phenocopies of the Creeper type. It is, of course, possible that other experimental procedures will result in such phenocopies. However, the fact that not every nonspecific growth restriction imposed on an early limb bud will produce automatically the Creeper symptoms seems to us of significance for the interpretation of the action of the Creeper factor.

Our data do not favor the assumption that the morphogenetic and histogenetic effects of the Creeper factor are secondary results of a primary growth restriction, as is implied in Landauer's hypothesis. They suggest an alternative explanation of its action, i.e., morphogenetic, histogenetic, and growth disturbances might be derived from a more basic deficiency. We agree with Landauer in his emphasis on the universal nature of the action of the Creeper gene and are inclined, therefore, to assume that we are dealing with some deficiency in a general metabolic or respiratory mechanism. This deficiency might concern a specific substance, or interfere with a specific reaction involved in such a general activity. The localized manifestations would be due to differential susceptibility of the various primordia for such a deficiency. This assumption would explain why the histogenesis of one type of tissue—chondrogenesis, for example—is impaired more seriously than the differentiation of other tissues, such as the musculature which seems to proceed normally even under the CpCp condition. Growth, morphogenesis, and histogenesis might be affected independently of one another and even at different stages of development. There is no difficulty in accommodating the experimental data of Fell and Landauer (1935) to such a scheme. The medium used in their tissue-culture experiments might have precluded the elaboration of a specific agent which is required for chondrogenesis. This effect might be quite independent of its growth-restricting effect.

While we find ourselves in agreement with Landauer's most recent discussion of the action of the Creeper factor (1941) in which he likewise considers specific deficiencies (in the elaboration of enzymes, etc.) operative in all parts of the embryo and throughout development, we cannot follow him in his emphasis on growth disturbances as their primary effects. However, it is evident that our present data are strongly suggestive of, but give no crucial evidence for, an independence of histogenetic and of growth disturbances.

LITERATURE CITED

David, P. R. 1936. Studies on the Creeper fowl. X. A study of the mode of action of a lethal factor by explantation methods. Arch. f. Entw'mech., **135**:521.

Dunn, L. C. 1927. The occurrence of chondrodystrophy in chick embryos. II. The genetic evidence. Arch. f. Entw'mech., **110**:341.

Fell, H. B., and Landauer, W. 1935. Experiments on skeletal growth and development *in vitro* in relation to the problem of avian phokomelia. Proc. Royal Soc. London, B, **118**:133.

Fugo, N. W., and Witschi, E. 1938. Removal of the hypophyseal primordium in the chick during the second day of incubation. Acta biol. Latv., **8**:73.

Hamburger, V. 1938. Morphogenetic and axial self-differentiation of transplanted limb primordia of two-day chick embryos. Jour. Exper. Zoöl., **77**:379.

———. 1939*a*. The development and innervation of transplanted limb primordia of chick embryos. *Ibid.*, **80**:347.

———. 1939*b*. A study of hereditary chondrodystrophy in the chick ("Creeper fowl") by means of embryonic transplantation. Proc. Soc. Exper. Biol. and Med., **41**:13.

Hamburger, V., and Waugh, Molly. 1940. The primary development of the skeleton in nerveless and poorly innervated limb transplants of chick embryos. Physiol. Zoöl., **13**:367.

Landauer, W. 1927. Untersuchungen über Chondrodystrophie. I. Allgemeine Erscheinungen und Skelett chondrodystrophischer Hühner-Embryonen. Arch. f. Entw'mech., **110**:195.

———. 1929. Untersuchungen über Chondrodystrophie. III. Die Histologie der Drüsen mit innerer Sekretion von chondrodystrophischen Hühner-Embryonen. Virchow's Arch., **271**:534.

———. 1931. Untersuchungen über das Krüperhuhn. II. Morphologie und Histologie des Skeletts. Zeitschr. f. mikr.-anat. Forsch., **25**:115.

———. 1932. Studies on the Creeper fowl. III. The early development and lethal expression of homozygous Creeper embryos. Jour. Gen. **25**:367.

———. 1933. Untersuchungen über das Krüperhuhn. IV. Die Missbildungen homozygoter Krüper-Embryonen auf späteren Entwicklungsstadien. Zeitschr. f. mikr.-anat. Forsch., **32**:359.

———. 1934. Studies on the Creeper fowl. VI. Skeletal growth of Creeper chickens. Storrs Agric. Exper. Stat. Bull. 193, p. 1.

———. 1939. Studies on the Creeper fowl. XII. Size of body, organs, and long bones of late homozygous Creeper embryos. *Ibid.* 232, p. 1.

———. 1941. Teratological correlations and the mechanism of gene expression. Proc. Seventh Internat. Gen. Cong. (Edinburgh, 1939). (In press.)

Landauer, W., and Dunn, L. C. 1930. Studies on the Creeper fowl. I. Genetics. Jour. Gen., **23**:397.

Rudnick, D., and Hamburger, V. 1940. On the identification of segregated phenotypes in progeny from Creeper fowl matings. Genetics, **25**:215.

PLATE I

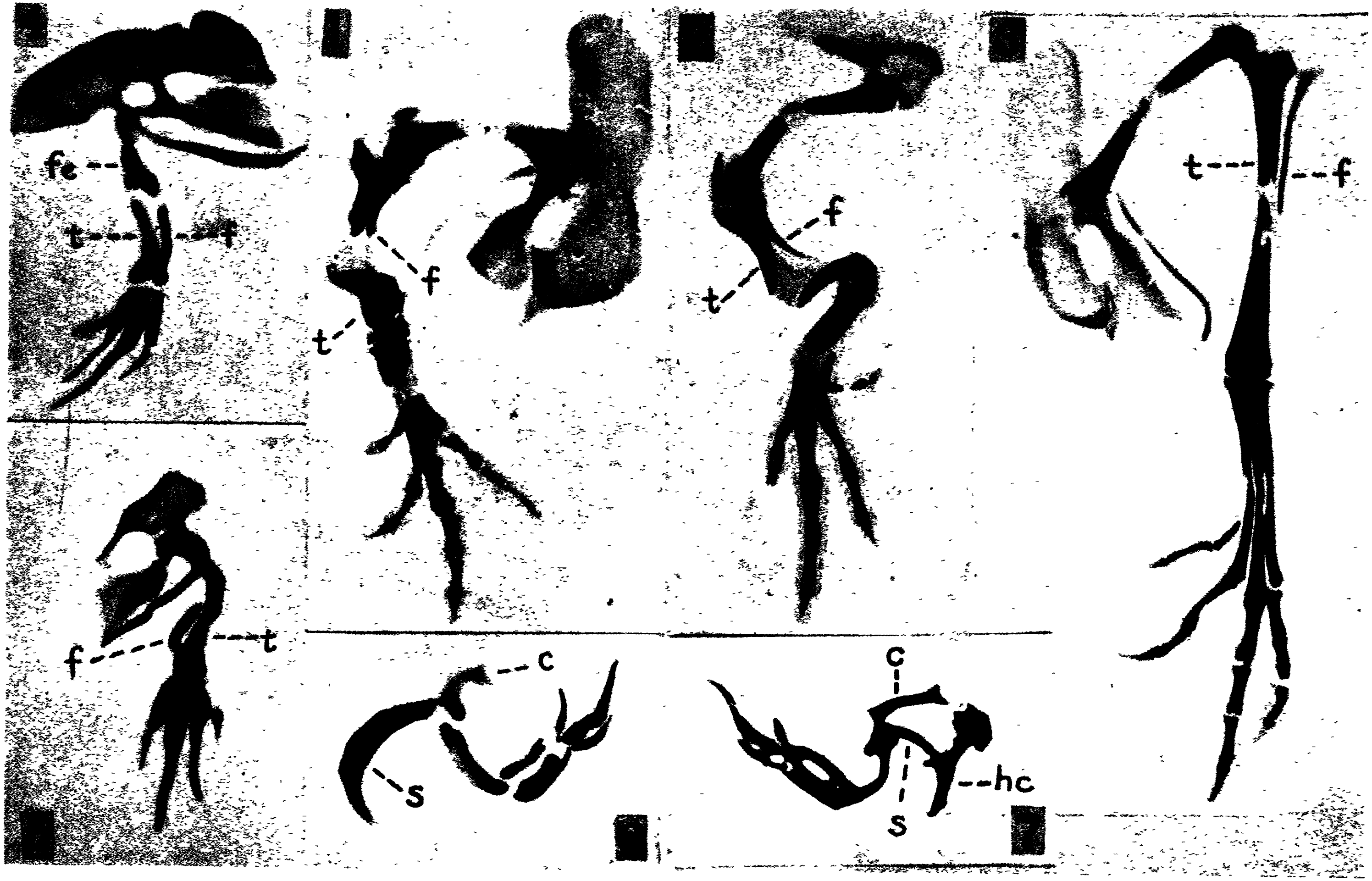

PLATE I

FIG. 1.—Cl 84. Left leg and pelvic girdle of chondrodystrophic (Cpcp) donor embryo. Fixed at 11 days of incubation. Methylene blue; ×6.3; *f* = fibula; *t* = tibia.

FIG. 2.—Cl 84. Right leg of the same embryo, transplanted in Stage 4 (small bud; 67 hours of incubation) to the flank of a normal embryo of the same stage. Fixed at 11 days of incubation. Methylene blue; ×6.3; *f* = fibula; *t* = tibia.

FIG. 3.—Cl 84. Control. Right leg of host of transplant in Fig. 2. Fixed at 11 days of incubation. Methylene blue; ×5.3; *f* = fibula; *t* = tibia.

FIG. 4.—Left leg of a phocomelic (CpCp) embryo, for comparison with Fig. 5. Fixed at 12 days of incubation. Methylene blue; ×5; *fe* = femur; *f* = fibula; *t* = tibia.

FIG. 5.—Cl 51. Right leg of an early lethal (CpCp) embryo transplanted in Stage 3 (very small bud; 64 hours of incubation) to the flank of a normal embryo of the same stage. Fixed at 12 days of incubation. Methylene blue; ×5.5; *f* = fibula; *t* = tibia.

FIG. 6.—Right wing of a phocomelic (CpCp) embryo, for comparison with Fig. 7. Fixed at 12 days of incubation. Methylene blue; ×5; *c* = coracoid; *s* = scapula.

FIG. 7.—Cl 96. Right wing of an early lethal (CpCp) embryo transplanted in Stage 1 (thickened somatopleure; 27 somites; 54 hours of incubation) into coelomic cavity of normal embryo, Stage 3 (very small wing buds; 68 hours of incubation). Transplant developed on inside of body wall, near right host wing. Fixed at 17 days of incubation. Methylene blue; ×3; *c* = coracoid; *hc* = host coracoid; *s* = scapula.

EFFECT OF THYMUS GLAND, THYMUS EXTRACTS, AND THYMUS DERIVATIVES ON GROWTH AND METAMORPHOSIS OF AMPHIBIAN LARVAE

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ALTHOUGH many years have elapsed since experiments were first performed on the thymus gland, the precise physiological role of this organ still has not been definitely established. Considerable interest in the problem has been re-aroused recently by the results obtained with Hanson's thymus extract by Rowntree and his associates (1936*a*, *b*), and with thymocrescin—another type of thymus extract—by Asher (1936) on the development of the rat. The major portion of the work has been conducted on mammals, and for a description of the literature in this field the reader is referred to the reviews by Anderson (1932), Gudernatsch (1937), and Nelson (1939). A considerable amount, however, has also been performed on amphibians. This is not surprising since, in such material, growth and differentiation processes may be favorably studied in large numbers of genetically similar animals in the comparatively short time of the metamorphic period. As in mammals, very few noncontroversial findings have emerged. Gudernatsch (1912, 1917) and Romeis (1914) reported retardation of metamorphosis and increased growth in developing larvae which were fed thymus substance. Since these effects were obviously opposite to those produced by thyroid material, a thymus-thyroid antagonism was postulated. On the other hand, Swingle (1917), Uhlenhuth (1919), Romeis (1925), Abderhalden (1926), and Janes and Segaloff (1938), employing either feeding or immersion techniques, indicated that the thymus gland plays no significant role in amphibian development.

Criticism, however, may be lodged against the methods employed by the foregoing investigators. The failure by some to obtain positive results with fed thymus material may possibly be due to a destruction of the active thymic principle, if it does exist, in the alimentary tract. The immersion method, likewise, may be criticized, since its use necessarily implies that the principles can be absorbed through the integument. In view of the recent success obtained with injections of thymus extracts in mammals, it was considered worth while to subject the problem in amphibia to an extensive reinvestigation using the more precise injection technique.

MATERIALS AND METHODS

Two series of experiments were performed, one on *Rana pipiens* and the other on *R. palustris*. *R. pipiens* eggs were secured from several healthy female frogs and fertilized according to the methods described by Rugh (1934). *R. palustris* eggs were obtained from a running stream in the open field.

In the first series of experiments approximately 800 *R. pipiens* tadpoles were used. They were immersed in tap water which was changed daily and kept at a temperature of 19°–22° C. Crowding effects were minimized by placing sets of 25 tadpoles into separate 2-liter glass bowls. The animals were divided into eight groups of approximately 100 animals each. Injections were begun at the hind limb bud stage.

Group I received injections of 0.05 cc. of a suspension containing the equivalent of one to two adult *R. pipiens* thymi ground in frog Ringer's solution. These glands were dissected from approximatley 2,000 frogs during the course of the experiment. They were usually injected within 1–2 hours after dissection. On certain occasions they were stored before being used for no longer than 24–48 hours in the freezing compartment of a refrigerator. The animals of Group II were injected with 1–2 mg. of thymocrescin[1] dissolved in 0.05 cc. Ringer's. Each milligram corresponded to 0.45–0.65 gm. of fresh calf thymus. Group III was given injections of 0.05 cc. of Hanson's thymus extract.[1]

In view of the fact that Schaffer *et al.* (1938) have recently claimed that Hanson's extract is rich in iodine-reducing compounds such as glutathione and cysteine, we thought it advisable to test these materials also. Consequently, the animals of Groups IV and V were injected, respectively, with 0.25–0.50 mg. glutathione (Hoffman–La Roche) and 0.25–0.50 mg. cysteine hydrochloride dissolved in 0.05 cc. Ringer's.

Groups VI, VII, and VIII served as controls. Group VI received injections of 0.05 cc. of a suspension containing 1–2 mg. of fresh frog skeletal muscle ground in Ringer's. Group VII comprised the untreated controls. Another untreated group (VIII) was employed to control the effects of Group III since unavoidably the Hanson's extract injections were begun approximately 3 weeks later than the others. All injections, in this first series, were administered twice a week for a period of 12 weeks.

In the second series approximately 450 *R. palustris* tadpoles were used. These were segregated into four groups and kept under conditions similar to those of the first series. Injections were again initiated at the hind limb bud stage. Group A received injections of 1–2 mg. thymocrescin in 0.05 cc. Ringer's, Group B, 0.05 cc. Hanson's extract, and Group C, 1–2 mg. crystalline egg albumin in 0.05 cc. Ringer's. Group D consisted of untreated controls. In this series each experimental animal received a total of 20 injections over a period of 8 weeks.

All materials, with the exception of Hanson's extract, were freshly prepared immediately before each injection. No metal instruments were used in handling the glutathione and cysteine. All injections were made intraperitoneally through a narrow glass capillary tube in the younger animals and through a 27-gauge hypodermic needle later in the experiment. After being injected, each animal was placed, for a few seconds, on its dorsal side on a piece of moistened absorbent cotton. In test animals, with the use of colored dyes, it was found that with this method very little, if any, of the injected material was lost upon reimmersing the animals.

In preliminary trial tests several of the substances employed, particularly Hanson's extract and cysteine hydrochloride, were found to be extremely toxic. This was due largely to the high acidity of these substances and so, in both series of experiments, all materials were brought to pH 6.5 immediately before injection. No precipitation occurred with any of the substances at this pH. With this procedure, only a very small number of deaths occurred in all the groups throughout the course of the experiment.

All animals were fed a diet consisting largely of boiled spinach, occasionally supplemented with boiled liver and rolled oats during the experimental period. It was considered likely that, if the various thymus substances, extracts, and derivatives employed exerted any truly endocrine effects, these should be revealed even though the animals were fed a normal diet.

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[1] We are greatly indebted to Mr. Albert Segaloff and Dr. Warren O. Nelson of the department of anatomy, Wayne University College of Medicine, for fresh thymocrescin made by Asher's method, and for Hanson's extract.

At certain intervals after initiation of treatment, the total (from snout to tip of tail), trunk (from snout to base of tail), and hind limb lengths of each animal were measured as accurately as possible to the nearest millimeter. The animals of each group were then collected in a net, and, after the excess water had been allowed to drip through the net, the total weights were determined, from which the average weight of the animals in the various groups could be computed. It is realized that weights determined in this way represent only an approximation. The nature of the experiment made it impracticable to utilize the dry-weight method. In any event, as will be seen below, the wet-weight data are in accord with other results on the same animals.

TABLE 1

EFFECT OF THYMUS MATERIALS ON DEVELOPMENT OF *Rana pipiens* LARVAE

Group No.	Treatment	Date	*TL*	*TrL*	*HL*	Percentage *M*	Weight (gm.)
I.	Frog thymus	March 19	70.3±1.39	24.8±0.39	10.1±0.72	0	3.2
		April 21	79.3 2.93	26.8 0.59	31.2 2.34	41.3	5.2
II	Thymocrescin	March 19	72.7 0.97	25.8 0.37	10.5 0.56	0	3.5
		April 21	79.9 1.50	26.8 0.37	30.3 1.50	56.0	4.8
IV.	Glutathione	March 19	70.6 1.17	24.7 0.39	8.7 0.46	0	3.1
		April 21	79.0 1.42	25.1 0.39	26.4 1.33	45.8	4.7
V.	Cysteine	March 19	69.8 1.33	23.8 0.48	10.4 0.70	0	3.2
		April 21	77.3 1.63	25.5 0.60	31.8 2.15	49.0	4.6
VI.	Muscle	March 19	64.8 1.32	22.8 0.44	7.8 0.64	0	2.6
		April 21	74.7 1.87	24.0 0.47	28.4 2.13	27.6	3.9
VII.	Untreated	March 19	72.6 0.99	25.2 0.41	8.9 0.51	0	3.7
		April 21	80.0±2.43	26.9±0.59	27.8±1.78	50.0	5.0

TL, *TrL*, and *HL* represent the mean total, trunk, and hind limb lengths (± standard error) of those animals not as yet exhibiting extruded fore limbs after 16 (March 19) and 24 (April 21) injections.

Percentage *M* represents the percentage of animals showing both fore limbs extruded.

Weight (gm.) is the mean wet weight (in grams) of animals not as yet exhibiting both fore limbs extruded.

RESULTS

In Table 1 may be seen the data for the first series of experiments after 16 and 24 injections had been administered to *R. pipiens* tadpoles. After 16 injections no appreciable differences could be noted in the total, trunk, hind limb lengths, or weights of the tadpoles in any of the groups, with the exception of those in the muscle group which appeared to be somewhat retarded in both growth and metamorphosis, the latter being judged at this time by hind limb length. None of the animals in any of the groups had extruded fore limbs at this time. Even after 24 injections, the total, trunk, hind limb lengths, weights, and percentage of the animals which had metamorphosed had not been significantly altered, with the exception again of those animals which had received the muscle injections. These had been delayed in their growth and differentiation processes.

Table 2 gives the results for Hanson's extract on *R. pipiens* tadpoles together with a group of untreated controls. After 10 injections, no significant differences in the two groups could be determined. By the eighteenth injection, however, the animals treated with Hanson's extract were more advanced in metamorphosis, as judged by hind limb length and percentage showing fore limbs, than were the untreated controls. The injected animals were also slightly larger in size and heavier. After 24 injections, 84.4 per cent of the treated animals had both fore limbs extruded, whereas only 63.3 per cent of the untreated controls had reached this stage of development.

TABLE 2

EFFECT OF HANSON'S EXTRACT ON DEVELOPMENT OF *Rana pipiens* LARVAE

Group No.	Treatment	Date	*TL*	*TrL*	*HL*	Percentage *M*	Weight (gm.)
III	Hanson's extract	March 19	68 6±0 80	25 0±0 30	8 1±0 34	0	3.0
		April 21	82.4 1 14	27 8 0 29	32 4 1.25	49 9	5 3
		May 12	.. . .	.... .	. . ...	84 4	....
VIII	Untreated	March 19	66 8 1 24	24 0 0 45	7 5 0 54	0	2 9
		April 21	79 4±1 50	25 2±0 48	24 6±1 71	33 3	4 3
		May 12	. . ..	. . . .	. . . . .	63 3	....

TL, *TrL*, and *HL* represent the mean total, trunk, and hind limb lengths (±standard error) of those animals not as yet exhibiting extruded fore limbs after 10 (March 19), 18 (April 21), and 24 (May 12) injections.

Percentage *M* represents the percentage of animals showing both fore limbs extruded.

Weight (gm.) is the mean wet weight (in grams) of animals not as yet exhibiting both fore limbs extruded.

TABLE 3

EFFECT OF THYMUS EXTRACTS ON DEVELOPMENT OF *Rana palustris* LARVAE

Group	Treatment	Date	*TL*	*TrL*	*HL*	Percentage *M*	Weight (gm.)
A....	Thymocrescin	July 13	54 3±0 75	17 6±0 21	9 7±0 84	0	1 4
		August 3	61.8 0 89	19 7 0 24	17 4 1 32	43 0	2.0
B......	Hanson's extract	July 13	56 9 0 72	18 4 0 18	11 0 0 75	0	1 5
		August 3	66.0 0 98	20 6 0 25	22 8 1 43	65 1	2 8
C......	Albumin	July 13	52 3 0 77	17 0 0 22	7 1 0.70	0	1.2
		August 3	58 5 1.02	18 3 0 27	15 0 1 24	22 2	1.4
D. ...	Untreated	July 13	55 0 0.89	17 6 0 22	9 0 0 82	0	1.4
		August 3	63.2±1 44	19.7±0.34	16 2±1.61	48 7	1 9

TL, *TrL*, and *HL* represent the mean total, trunk, and hind limb lengths (±standard error) of those animals not as yet exhibiting extruded fore limbs after 13 (July 13) and 20 (August 3) injections.

Percentage *M* represents the percentage of animals showing both fore limbs extruded.

Weight (gm.) is the mean wet weight (in grams) of animals not as yet exhibiting both fore limbs extruded.

In Table 3 are listed the results for the second series of experiments. The data corresponding to 7 injections of the various materials are not given since these showed that no significant effects had as yet been obtained. After 13 injections, however, several slight differences between the various groups were noted. The tadpoles receiving the albumin injections were somewhat retarded in general body growth and degree of metamorphosis, whereas those to which Hanson's extract was administered were, on the whole, slightly more advanced in metamorphosis, although their general body size and weight were not significantly different from those of the untreated controls. Thymocrescin was without any effect at this time. After 20 injections, the differences between these groups were more marked. Crystalline egg-albumin injections had produced a definite delay in metamorphosis and an inhibition in growth. The animals treated with Hanson's extract were, as in the first series of experiments, slightly larger in size and heavier and definitely more advanced in metamorphosis as seen both from the number of animals which exhibited fore limbs and from the hind limb lengths of the remaining animals. The thymocrescin-injected animals showed measurements similar to those of the untreated controls.

DISCUSSION

It would appear from the above results that among the substances employed the only one capable of causing an increase in the growth and rate of metamorphosis of *Rana* tadpoles is Hanson's extract. The effect on growth is slight, whereas the influence on differentiation is more marked. Both processes are apparently affected to a greater extent in the later stages of metamorphosis. If the effects obtained in the present experiments with Hanson's extract are to be interpreted in terms of its sulphhydryl content (Rowntree *et al.*, 1938) it is difficult to see why such substances as glutathione and cysteine are without influence on the development of the metamorphosing anuran. Since, however, Rowntree *et al.* have claimed that the effect of such compounds is not so great as that of the whole Hanson's extract, one might assume that the sulphhydryl in the extract is present in some more potent form, perhaps linked with some other organic component, or that other substances present in the extract also contribute to the activity. There is also the possibility that, in our experiments, glutathione and cysteine were not administered in a dosage necessary to give rise to the effect.

The effect of the Hanson's extract may, on the other hand, be purely a nutritional one. The diet which was administered to all the animals throughout the experiment was quite adequate in that the untreated controls, as a whole, metamorphosed without any apparent abnormalities. All animals in this group, both in the early and in the later stages of metamorphosis, were healthy and vigorous. The diet, however, may not have been an optimal one and may have been supplemented by the addition of the nutritive materials present in the extract.

The reason why Hanson's extract produces a relatively greater effect in the later stages of metamorphosis is also difficult to understand. If it acts synergistically with the thyroid as some have claimed (Colosi, 1933) or antagonistically toward it as others have reported, one would expect some effect to be exerted in the earlier stages also. Moreover, as is well known, thyroid substance causes a more rapid metamorphosis at the expense of growth, whereas Hanson's extract, as was seen, causes an acceleration of both processes.

It is to be emphasized that the effects obtained with Hanson's extract, although significant, are slight in degree and hardly endocrine-like in nature. If it were possible to rear and inject amphibians through successive generations as Rowntree and his associates have done with rats, the effects of the extract might have been augmented.

The results also indicate quite clearly that no significant effects were obtained with thymocrescin or ground frog-thymus material. It is interesting to note that, although thymocrescin contains labile sulphur, recent analyses by Segaloff (quoted by Nelson, 1939) have shown it to be devoid of compounds with the sulphhydryl linkage, and this may account for its inability to affect the development of anurans. Our original purpose in employing frog thymus was based largely on analogy with the methods used by several investigators who showed that injections of homozoic pituitary substance are more effective than that of other species in inducing developmental processes in the amphibia (Rugh, 1935). No such endocrine influence, however, was obtained in our experiments with frog thymus. The possibility still remains that some effect on development might have occurred if extracts of the glands, prepared by the methods of Hanson, had been employed.

It was believed at first that the crude nature of the protein in the injected frog-muscle material was the cause for its observed inhibitory effect on tadpole development. As can be seen from our results, however, injections of crystalline egg albumin—a purified protein—produced the same effect. We are, at the present, unable to explain this inhibitory influence of frog muscle and crystalline egg albumin on the development of anuran larvae.

SUMMARY

1. Intraperitoneal injections of Hanson's thymus extract produced a significant, although slight, increase in the growth and differentiation of *R. pipiens* and *R. palustris* larvae reared on a normal diet.
2. Injections of adult *R. pipiens* thymi, glutathione, and cysteine hydrochloride were without effect on the development of *R. pipiens* larvae.
3. Thymocrescin failed to exert any significant effect in both species of *Rana*.
4. In *R. pipiens*, frog-muscle injections were found to cause a delay in growth and differentiation. This same effect was obtained in *R. palustris* with crystalline egg albumin.
5. On the basis of these results, it is possible to conclude that thymus substance, extracts, or derivatives do not exert any obvious hormonic influence on growth and differentiation of developing anuran larvae.

LITERATURE CITED

ABDERHALDEN, E. 1926. Über das Wesen der Wirkung der Verfütterung von Thymusgewebe auf Wachstum und Entwicklung von Froschlarven. Pflüger's Arch. f. d. ges. Physiol., 211:324.

ANDERSON, D. H. 1932. The relation between the thymus and reproduction. Physiol. Rev., 12:1.

ASHER, L. 1936. Untersuchungen der hormonalen Funktion der Thymus. Handb. d. biol. Arbeitsmethoden, 5:929.

COLOSI, G. 1933. Action of thymus feeding on metamorphosis of anurans. Boll. Soc. nat. Napoli, 44:115.

GUDERNATSCH, F. 1912. Fütterungsversuche an Amphibienlarven. Zentralbl. f. Physiol., 26:323.

———. 1917. Studies on internal secretion. V. Treatment of tadpoles with thyroid and thymus extracts. Anat. Rec., 11:357.

-. 1937. The present status of the thymus problem. A review of nonsurgical experimentation in this field. Med. Rec., 146:101.

JANES, R. G., and SEGALOFF, A. 1938. Growth and metamorphosis of anuran larvae on thymus extracts. Proc. Soc. Exper. Biol. and Med., 39:172.

NELSON, W. O. 1939. The relation of the thymus and pineal glands to genital function. In Sex and internal secretions (2d ed. [Williams & Wilkins Co.], p. 1121).

ROMEIS, B. 1914. Experimentelle Untersuchungen über die Wirkung innersekretorischer Organe. II. Der Einfluss von Thyroidea- und Thymusfütterung auf das Wachstum, die Entwicklung und die Regeneration von Anurenlarven. Arch. f. Entw'mech. d. Org., **40**:571.

———. 1925. Die Wirkung der Verfütterung frischer Thymus auf Froschlarven. Arch. f. mikr. Anat. u. Entw'mech., **104**:273.

ROWNTREE, L. G.; CLARK, J. H.; STEINBERG, A.; EINHORN, N. H.; and HANSON, A. M. 1936*a*. Further studies on the thymus and pineal glands. Penn. Med. Jour., **39**:603.

———. 1936*b*. The role of the thymus and pineal glands in growth and development. N.Y. State Jour. Med., **36**:1277.

ROWNTREE, L. G.; STEINBERG, A.; EINHORN, N. H.; and SCHAFFER, N. K. 1938. Certain iodine-reducing substances of thymus extract. Biological considerations. Endocrinology, **23**:584.

RUGH, R. 1934. Induced ovulation and artificial fertilization in the frog. Biol. Bull., **66**:22.

———. 1935. Pituitary-induced sexual reactions in the anura. *Ibid.*, **68**:74.

SCHAFFER, N. K.; ZIEGLER, W. M.; and ROWNTREE, L. G. 1938. Certain iodine-reducing substances of thymus extract. Chemical analysis of extract. Endocrinology, **23**:593.

SWINGLE, W. W. 1917. Feeding thymus to frog larvae. Biol. Bull., **33**:116.

UHLENHUTH, E. 1919. Function of thymus gland. Endocrinology, **3**:285.

INHIBITION OF LACTATION IN THE RAT[1]

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LITERATURE on the endocrine regulation of the mammary gland has been reviewed by Nelson (1936) and Turner (1939). It suffices to mention here that mammary growth is dependent upon ovarian secretions, which exert their characteristic effect only if the anterior pituitary is intact. Once structurally developed, the mammae secrete milk when stimulated by an anterior lobe lactogenic hormone. This latter hormone in turn exerts its characteristic function only when a concurrent adrenotropic-adrenal cortical activity is present (reviewed by Gaunt, 1937).

Still unsettled is the problem as to how lactogenic hormone secretion, and hence milk secretion, is regulated. In some forms at least nervous influences are involved (Selye, 1934; Ingelbrecht, 1935; Herold, 1939), and certainly endocrine factors are concerned.

Nelson, working primarily on the guinea pig, developed the theory that the secretion of estrogen by the ovary and placenta was a factor normally holding lactation in abeyance during pregnancy. This was thought to be accomplished by a twofold action: first, a direct action on the mammary glands and, second, a prevention of lactogenic hormone secretion. Folley and Kon (1938) suggested that any substance which stimulates mammary growth inhibits lactation. Many have found in various species (references by Folley and Kon, 1938) an inhibition of lactation by estrogens. The dosage needed, however, probably varies greatly with the species.

The presence or absence of an ovary, in the rat at least, modifies the suppressing action of estrogen on lactation, but opinions differ as to the extent of this ovarian influence (Anselmino and Hoffman, 1936; Folley and Kon). De Jongh's (1933) experiments indicated that the well-known inhibiting effects of gonadotropins on lactation might not be entirely through their stimulation of ovarian activity, since chorionic gonadotropin stopped lactation in ovariectomized as well as in normal mice. This suggests the converse of the mechanism described by Bates, Riddle, and Lahr (1937) in which lactogenic hormone suppressed production of gonadotropin in birds.

We report here a further investigation of endocrine factors which inhibit lactation and of conditions modifying their action in rats. An abstract of this work has been published (Edelmann and Gaunt, 1940).

METHODS

The amount of lactation of the experimental rats was judged by the growth rate of their litters, reduced to 4 young each. Hormone injections were begun on the day of delivery if not otherwise stated. Ovariectomy was performed on the day of delivery. Injections were generally carried out for 15 days, the age at which the young of normal litters began to eat. Controls consisted of 15 normal untreated mothers with litters reduced either to 4 or to 6 members. Such differences in litter size did not cause results to vary. One death occurred in the control group of 80 suckling young.

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RESULTS[2]

Chorionic gonadotropin (*Table 1, Ser. 1*).—The pregnancy urine hormone stimulates mammary development in intact rats; and ovariectomy in such stimulated animals is followed by a spontaneous milk secretion (Selye, Collip, and Thomson, 1933). It has been reported (Cannon, 1938) and denied (Hathaway, Davis, Reece, and Bartlett, 1939) that this hormone by itself has a marked inhibitory effect on established lactation in rats, although the latter authors found it to augment the lactation-inhibiting action of estrogen (Reece *et al.*, 1940). In our hands it was without appreciable effect in 6 intact rats in doses of 100–500 RU daily if given for not more than 5 days. With a 15-day injection period there was some inhibition in two cases. This may or may not have been a specific action of the hormone since Hathaway *et al.* found such extracts somewhat toxic in large doses.

We were more interested in the report of De Jongh that APL would inhibit lactation in castrate as well as in normal mice. With castrate rats, given doses of 100 RU per day for 15 days, an inhibition of lactation was found in only one of four cases, and this in view of the more extensive negative results with pregnant mare serum was probably not significant. None of the young died.

Pregnant mare serum (*Table 1, Sers. 2, 3, 4*).—PMS in doses of 0.5–1.0 cc. per day was the most effective inhibitor of lactation we have used. It gave a relatively quick response and resulted in the death of many young. In castrate animals, however, it was entirely without effect, indicating that a high level of gonadotropin is not per se detrimental to the release of lactogenic hormone. The inhibitory effect in intact animals was more marked if treatment was started 5 days before delivery.

Anterior pituitary extract (*Table 1, Sers. 5, 6*).—Gaunt and Tobin (1936) found in lactating adrenalectomized rats given cortical extract that lactation was diminished by the injection of anterior pituitary extract (Squibb). It was suggested that this inhibition was due to the estrin-producing properties of gonadotropins in the extract, despite the fact that the latter were slight in amount. When tested here in normal animals, intact or castrate, it had no effect on lactation. Its effect on adrenalectomized animals must have been in some way dependent upon the peculiar condition of those animals.

Estrogenic hormone (*Table 1, Sers. 7–13*).—Estrone (Amniotin) in doses of 200 IU per day inhibited lactation in intact animals but was without effect in castrates in doses of 300–5,000 IU per day. This finding is in apparent agreement with Anselmino and Hoffman (1936).

Using larger doses of estrogen (1 mg. per day of either estradiol-17-propionate or stilboestrol), we obtained an inhibition of lactation such as was obtained by Folley and Kon in castrate animals, but distinctly less than that obtained with similar doses in in-

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[2] The endocrine products used here were generously supplied us as follows: chorionic gonadotropin (Follutein), Anterior Pituitary Extract, Amniotin and stilboestrol by Dr. J. A. Morrell, E. R. Squibb & Sons; pregnant mare serum (Gonadin Serum) by Mr. Donald Wonder, Cutter Laboratories; testosterone propionate (Oreton) and progesterone (Proluton) by Dr. Erwin Schwenk, Schering Corporation; and estradiol-17-propionate (Follacro) by Dr. E. W. Blanchard, Schieffelin & Co.

TABLE 1

MEAN FIGURES ON THE GROWTH RATES OF LITTERS OF RATS AFTER VARIOUS TREATMENTS OF THE MOTHER

Treatment and Condition of Mother	No. Cases	On Fifth Day		On Ninth Day		On Thirteenth Day		On Sixteenth Day		Weight* Change in Mothers (Gm.)
		Weight Surviving Young (Gm.)	Percentage Dead	Weight Surviving Young (Gm.)	Percentage Dead	Weight Surviving Young (Gm.)	Percentage Dead	Weight Surviving Young (Gm.)	Percentage Dead	
1. Chorionic gonadotropin, 100 RU/day, castrate. .	4	9.1	0.0	13 3	0.0	17 8	0.0	20.0	0.0	− 6.8
2. Pregnant mare serum, 0.5–1.0 cc./day, intact...... ..	7	10 3	0.0	11.5	7.2	12.4	46 4	14.1	71.4	+11 0
3. Pregnant mare serum, 1 cc./day, castrate..	4	10 7	0 0	17 3	0.0	23 0	0.0	......	0.0	+18.0
4. Pregnant mare serum, 0.5 cc./day, intact, pretreated†	2	5 3	50 0	6 0	75 0	.....	100 0	.. ...	.	....
5. AP extract, 1–2 cc./day, intact.....	7	10 9	0 0	16 1	0.0	22 5	0 0	28.0	0.0	+25 0
6. AP extract, 1–2 cc./day, castrate...	4	11 3	0 0	19.1	0 0	25.6	0 0	30 0	0.0	+ 6.3
7. Amniotin, 200 IU/day, intact	5	11 8	0 0	15 1	0 0	15.9	25 0	20.7	45 0	+ 9.3
8. Amniotin, 300–5,000‡ IU/day, castrate..........	8	13 4	0 0	19 7	0 0	24 6	0 0	.. .	0.0	− 8 0
9. Amniotin, 200 IU/day, intact, pretreated§	4	9 6	0.0	10 0	25.0	11.1	56.3	.....	.....	0 0
10. Estradiol-17-propionate, 1 mg./day, intact	3	9 7	0 0	9 0	16 6	12 8	50 0	16.5	66 6	−10.3
11. Estradiol-17-propionate, 1 mg./day, castrate . . .	5	10 7	0 0	13 5	0 0	16 3	0.0	18 5	0 0	−14.2
12. Stilboestrol, 1 mg./day, intact	6	9 7	0.0	10 1	0 0	10.7	45.0	15 2	75 0	−16.3
13. Stilboestrol, 1 mg./day, castrate...	7	9 3	0 0	11.9	4 1	15.0	25 0	17 1	33.3	−12 5
14. Testosterone propionate, 3 mg./day, intact	5	7 5	0 0	10.8	50 0	11.5	56 2	13 3	75 0	+ 7 0
15. Testosterone propionate, 3 mg./day, castrate.......	4	9 7	0 0	13.4	8 3	15 8	8.3	16.9	8.3	+14.0
16. 1 mg. stilboestrol+3 mg. testosterone propionate, castrate................	1	4.0	0.0	5.0	0 0	......	100.0	......	.. .	+12.0
1 mg. stilboestrol+3 mg. testosterone propionate, castrate.e........	1	10.0	0 0	15.7	0.0	17.3	0.0	20.5	0 0	0 0
17. 1 mg. stilboestrol + 2 mg. progesterone, castrate ...	1	10 2	0.0	11 5	0.0	12.0	0.0	13.0	25.0	0.0
1 mg. stilboestrol+2 mg. progesterone, castrate....	1	10.0	0.0	9.5	0.0	.....	100 0	.....	.....	0.0
Controls, untreated..........	15	9.0	0.0	14.5	0 0	20.1	1.2	25.0	1.2	+ 5.0

* Weight change during injection period only.

† Treatment started 5 days before delivery.

‡ One received 300, 2 received 500, 1 received 1,000, 1 received 200, and 3 received 5,000 IU per day. No difference was found for the different dosages.

§ Treatment started 4 days before delivery.

tact mothers. If estrogen administration was started 4 days before delivery, the lactation-inhibiting response was considerably augmented.

Testosterone propionate (*Table 1, Sers. 14 and 15*).—Male hormone in doses of 3 mg. per day inhibited lactation markedly in intact mothers, as found by Folley and Kon (1938) and by Robson (1937), but like the estrogens it was much less effective in castrates.

Hormone combinations (*Table 1, Sers. 16 and 17*).—In two cases 1 mg. of stilboestrol was combined with 3 mg. testosterone propionate as a daily dose, and in two cases a similar amount of stilboestrol was given together with 2 mg. progesterone. Since the individual members of each pair responded differently, the cases are tabulated separately. There was, however, probably a greater inhibition than would have been expected from stilboestrol alone in such cases. Opportunity for performing conclusive experiments on this point with progesterone, the more significant substance, has not been available.

Weight change in mothers.—The effects of the various substances injected on the body weight of the mothers are probably of some significance as indicated below. Because of considerable variability, however, they must be interpreted cautiously. The great majority of our normal rats gained a little weight during the first 15 days of their lactation period. With large doses of estrogen a gain of weight never occurred and definite weight losses were the rule. Such results are entirely compatible with the well-known weight-reducing effect of these substances in nonlactating animals (references by McEuen, Selye, and Collip, 1937; and Folley and Kon, 1938). Testosterone-treated animals, on the other hand, never lost weight and in nearly every case gained—again as expected from the experiments of the above workers. Combinations of either testosterone or progesterone with stilboestrol seemed to cancel out the weight-reducing action of the latter, as Nelson (1938) and Korenchevsky, Hall, and Burbank (1939) found under other circumstances. Weight gains with anterior pituitary extract were to be expected since the preparation was rich in growth hormone. With pregnant mare serum there was in no case a weight loss and, on the average, weight gains as great as or greater than those in untreated animals. This indicates that if any sizable amount of estrogen was released as a result of this treatment it was counteracted by a simultaneous production of other steroids.

DISCUSSION

It is clear that the lactation-inhibiting properties of gonadotropins are mediated entirely through the ovary, but the absence of weight loss in mothers suggests that nonestrogenic steroids may be involved. Unless large doses are given, estrogen by itself—i.e., in castrate animals—is not a potent inhibitor of lactation in the rat. In intact animals it is much more effective, suggesting that under estrogenic stimulation the ovary releases additional substances which assist in the inhibition of lactation. A dose of estrogen can be found which decreases lactation only in intact animals, thus explaining the results of Anselmino and Hoffman, who detected no effects in castrates. The greater inhibition in intact animals is probably associated with the well-known luteinizing action of estrogen on rat ovaries (Hohlweg, 1934). The additional material secreted by such ovaries might be progesterone, a substance which, however, by itself does not stop lactation in doses of 1 mg. per day (Folley and Kon). It could, however, under physiological conditions act synergistically with estrogen or act in some way to prevent lactation initiation without the ability to inhibit an established milk secretion as suggested by Anselmino, Herold, Hoffman, and Pencharz (1936). Ovarian androgens could possibly be involved, since they are released in detectable amounts in various experimental conditions (Hill, 1937; Papanicaloau and Falk, 1938; Greene and Burrill, 1939).

An alternative explanation has been suggested to us (by Dr. Hans O. Haterius) to explain the greater effectiveness of estrogen in the intact as compared to the castrate animal. In the intact rat the liver, engaged in the inactivation of endogenous estrogen, may be less able to handle the large additional amounts given here and hence is more responsive than the castrate.

Results and conclusions given here must be limited to rats. In other species, such as the guinea pig, it takes less estrogen to suppress lactation, and there is a shorter latent period before any effects of treatment started at delivery can be observed (Nelson, 1934). The large doses of estrogen demanded by the rat for the inhibition of lactation and the long latent period for any effect raises question as to its physiological importance during gestation in this species (see also Turner, 1939). If the ovary has such a physiological role in the rat, it may be partially by means of some steroid other than estrogen, and we prefer such an interpretation of results found here.

SUMMARY

1. Chorionic gonadotropin did not inhibit lactation in castrate rats.
2. Pregnant mare serum inhibited lactation markedly in intact rats, but not at all in castrates.
3. An anterior pituitary extract that inhibited lactation in adrenalectomized rats did not do so in normal animals.
4. Estrogens in doses of 1 mg. per day inhibited lactation in both intact and castrate rats, but much less so in the latter. A dose could be found which would inhibit lactation in intact animals only.
5. Testosterone propionate acted like the estrogens and was a more effective lactation inhibitor in intact than in castrate animals.
6. In a limited series combinations of estrogen and progesterone or testosterone did not give decisive results.
7. Indirect evidence is given that when the ovary is stimulated by gonadotropin or estrogen it releases nonestrogenic substances which assist in the inhibition of lactation. The physiological importance of any direct action of estrogen in inhibiting lactation in the rat is questioned.

LITERATURE CITED

Anselmino, K. J., and Hoffman, F. 1936. Studien zur Physiologie der Milchbildung. Ueber die Laktationshemmung durch Follikelhormon. Zentralbl. f. Gynäk., 69:501.

Anselmino, K. J.; Herold, L.; Hoffman, F.; and Pencharz, R. U. 1936. Studien zur Physiologie der Milchbildung. Ueber den Hemmungstoff der Milchbildung. Zentralbl. f. Gynäk., 60:7.

Bates, R. W.; Riddle, O.; and Lahr, E. L. 1937. The mechanism of the antigonad action of prolactin in adult pigeons. Amer. Jour. Physiol., 119:610.

Cannon, F. E. 1938. Effect of anterior pituitary-like hormone on lactation in the albino rat. Proc. Soc. Exper. Biol. and Med., 37:52.

Edelmann, A., and Gaunt, R. 1940. Inhibition of lactation in the rat. Proc. Soc. Amer. Physiol. Soc., p. 54.

Folley, S. J., and Kon, S. K. 1938. The effect of sex hormones on lactation in the rat. Proc. Roy. Soc., London, B, 124:476.

Gaunt, R. 1937. The adrenal-pituitary relationship. Cold Spring Harbor Symp. on Quant. Biol., 5:395.

GAUNT, R., and TOBIN, C. E. 1936. Lactation in adrenalectomized rats. Amer. Jour. Physiol., 115:588.

GREENE, R. R., and BURRILL, M. W. 1939. Androgenic function of APL stimulated ovaries in immature rats. Proc. Soc. Exper. Biol. and Med., 42:761.

HATHAWAY, I. L.; DAVIS, H. P.; REECE, R. P.; and BARTLETT, J. W. 1939. Effect of pregnancy urine extract on lactation in the rat. Proc. Soc. Exper. Biol. and Med., 40:214.

HEROLD, L. 1939. Einfluss der Hypophysenstieldurchtrennung auf die Lactation. Arch. f. Gynäk., 168:534.

HILL, R. T. 1937. Ovaries secrete male hormone. Endocrinology, 21:495.

HOHLWEG, W. 1934. Veränderungen des Hypophysenvorderlappens und des Ovariums nach Behandlung mit grossen Dosen von Follikel-Hormon. Klin. Wchnschr., 13:92.

INGELBRECHT, P. 1935. Influence du système nerveaux central sur la mamelle lactante chez le rat blanc. Compt. rend. Soc. biol., 120:1369.

JONGH, S. E. DE. 1933. Weitere Untersuchungen über Laktationshemmung. Acta brev. Neerl., 3:88.

KORENCHEVSKY, V.; HALL, K.; and BURBANK, R. 1939. The manifold effects of prolonged administration of sex hormones to female rats. Biochem. Jour., 33:372.

MCEUEN, C. S.; SELYE, H.; and COLLIP, J. B. 1937. Effect of testosterone on somatic growth. Proc. Soc. Exper. Biol. and Med., 36:390.

NELSON, W. O. 1934. Studies on the physiology of lactation. III. The reciprocal hypophyseal-ovarian relationship as a factor in the control of lactation. Endocrinology, 18:33.

———. 1936. The endocrine control of the mammary gland. Physiol. Rev., 16:488.

———. 1938. Some effects of androgens and estrogens on the reproductive organs and hypophyses of adult male rats. Proc. Amer. Physiol. Soc., p. 154.

PAPANICOLAOU, G. N., and FALK, E. A. 1938. The production of general muscular hypertrophy by the androgenic hormone. Anat. Rec., Suppl., 70:61.

REECE, R. P.; BARTLETT, J. W.; HATHAWAY, I. L.; and DAVIS, H. P. 1940. Hormonal inhibition of lactation. Proc. Soc. Exper. Biol. and Med., 43:183.

ROBSON, J. M. 1937. Action of testosterone on lactation. Proc. Soc. Exper. Biol. and Med., 36:153.

SELYE, H. 1934. On nervous control of lactation. Amer. Jour. Physiol., 107:535.

SELYE, H.; COLLIP, J. B.; and THOMSON, D. L. 1933. Anterior pituitary and lactation. Proc. Soc. Exper. Biol. and Med., 30:588.

TURNER, C. W. 1939. The mammary glands. Chap. xi in Sex and internal secretions. Baltimore: Williams & Wilkins.

EXPERIMENTAL MODIFICATION OF BREEDING CYCLES IN GOATS[1]

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RECENT studies on the modification of sexual or breeding cycles of field mice, ferrets, raccoons, guinea pigs, ground squirrels, cottontail rabbits, and other mammals with limited annual breeding periods confined to the seasons of long or lengthening days, shows that some, but not all of them, are sexually photoperiodic. Within limits, the breeding cycles of the sexually photoperiodic ones can be modified at will by altering experimentally the duration of their daily periods of exposure to light (Bissonnette, 1936, 1938; Marshall, 1936, 1937; Wells and Zalesky, 1940; and others cited by them).

Some animals, however, breed not in the long-day seasons but in those of short or shortening day. Brook trout, sheep, deer, and goats are some of these. The change-over of the breeding seasons of the three last-named species when they are transferred to the Southern Hemisphere (Marshall, 1937), and the success of Hoover and Hubbard (1937) in causing a December-spawning strain of brook trout to spawn and produce viable, well-growing, healthy offspring in August, suggested that similar success might be obtained with goats. Such control and shifting of breeding cycles will make possible the "freshening" of some members of large flocks throughout the year. It would enable breeders to bridge the hiatus which normally occurs from late August to late January, because goats have a gestation period of 21–22 weeks and fail to breed between early April and September.

The hope of success led to the experiment to be described here, planned and carried out jointly at the Hillshires Farm, Killingly, Connecticut, with the aid and supervision of Emil Kaiser, chief herdsman and technician there.

MATERIALS AND METHODS

Four female Toggenburg goats and one female Nubian goat were housed in a small room off the main stable for females, in which about one hundred and forty others served as controls under exactly similar conditions of feeding, care, and ventilation. Both groups were turned out to pasture by day when the weather was suitable. Daily periods of pasturing conformed to the schedules of lighting of the two groups.

One Toggenburg male was confined in a separate stable about 100 yards from the stables for females, under feeding and lighting conditions similar to those of experimental females.

Controls were subject to natural conditions of lighting throughout the experiment except for being brought into the well-lighted stable at the usual hours. These hours varied somewhat with the seasons and weather and were the same as those for the experimental animals except as stated below.

Experimental animals received normal daylight throughout the early part of the experiment with light added at night from a 100-watt bulb, placed in front and a little above them in such position that they could not hide their eyes from its direct rays when it was lighted. During later parts of the experiment they were subject to reduction of daily periods of daylight below normal for the season by being driven into their stable at times specified and the room darkened by light-tight shutters over its windows. This, of course, made their nights during this part of the schedule periods of total darkness unaffected by moonlight or evening or morning twilights. The electric lights in the two experimental stables were wired on the same circuit controlled by an electric time switch. This was set to give the schedule of added electric light for the days following, up to May 24 (Table 1). From that date onward the animals were driven into the stable before sunset and the blinds closed to reduce the daily period of light below normal (Table 1), on a schedule reducing it 1 hour each week to 6 hours on June 28. This period was then adhered to until July 5, when the experimental animals were all turned out to pasture and left day and night with the male. During the period of reduced lighting, the blinds were opened during the night before morning twilight each night, so that reductions were made only at one end of the day until June 28, after which blinds were opened at 6:00 A.M. After July 5, lighting was normal for the season.

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[1] Aided by grants from the Penrose Fund of the American Philosophical Society, 1938–40, and by goats and their care and keep from Hillshires Farm, Killingly, Connecticut.

TABLE 1

TIME SCHEDULE FOR EXPERIMENTAL GOATS

Date of Change of Time	Time Switch On	Added Hours of Light (+) or Hours Less (−)	Time Switch Off (S) or Blinds Closed (B)
Jan. 25	5 05 P.M. E.S.T.	+1	6 05, S P.M. E.S.T.
Feb. 4	5.17	+2	7.17, S
14	5.30	+3	8.30, S
24	5.42	+4	9 42, S
Mar. 6	5 54	+5	10 54, S
16	6.06	+6	12.06, S
26	6 18	+7	1 18, S A.M.
Apr. 5	6.29	+6	12 29, S P.M.
12	6 37	+5	11 37, S
19	6.44	+4	10 44, S
26	6.52	+3	9 52, S
May 3	7 00	+2	9.00, S
10	7.08	+1	8.08, S
17	7.15	0	....
24	8 22 P.M. D.S.T.	−1	7.22, B P.M. D.S.T.
31	8.28	−2	6.28, B
June 7	8.33	−3	5 33, B
14	8 37	−4	4.37, B
21	8.40	−5	3.40, B
28	8 40	−6	3.15, B to 6 00 A.M.
July 5	8 40	−6	3.15, B to 6 00 A.M.
6	All on normal schedule and out in pasture day and night		

The females used were numbered 38T, 39T, 40N, 49T, and 52T, the "T" and "N" signifying Toggenburg and Nubian, respectively. All remained in good health throughout the months (over a year) during which they were under observation.

OBSERVATIONS AND RESULTS

Controls showed latest heat periods on or before March 15 and no normal ones thereafter until after September 1. Occasional very short and weak signs—almost, if not quite, unnoticeable and too short for breeding unless the animals were running continuously with the male—occurred, but they were of too short duration to last until the female could be taken to the male. Such heat periods are seldom, if ever, effective in producing offspring.

TABLE 2

HEAT PERIODS OF EXPERIMENTAL FEMALES BETWEEN FEBRUARY AND JULY 5*

Animal	First Heat	Second Heat	Tried with ♂	Reaction	Remarks
38T . .	. .	. .	. . .	.	No heat
39T	May 17	June 14	June 14	Mated	♂ not aggressive
40N	May 29	June 16, 17	June 16, 17	Not mated	♂ not interested
49T .	.	.		.	No heat
52T .	May 28				No heat

* None of the heat reactions were normal, as they occurred in autumn The male mated but once with one female and was not interested later

TABLE 3

MATINGS AND RESULTS SUBSEQUENT TO JULY 5
AS SHOWN BY BIRTHS IN DECEMBER

Animal	Mating Date	Delivery	Sex of Kids	Milk Record
38T .	July 15	Dec. 15	2♂♂	Good milker
39T		. . . . . .	. . .	No milk
40N .	July 14	Dec. 14	1♂+1♀	Light milker
49T	July 16	Dec. 16	1♂	Good milker
52T	July 11	Dec. 11	1♂+1♀	Light milker

Experimentals were not noticed in heat after some dates in February until 39T showed the twitching of the tail and receptivity that characterize heat, on May 17. Two others showed signs of heat in May, and two of these three females again showed heat signs in June, at which times they were tried with the experimental male and one was served, or mated (Table 2). None of the heat reactions occurring in May and June with these females was normal in intensity or completeness, and the male was not very aggressive in his attempts to mate.

After being turned out to continuous pasture with the male, four of the females must have mated at least once and probably more times, for in December they gave birth to kids, as shown in Table 3 along with the calculated probable dates of effective matings and the number and sex of the kids born.

DISCUSSION AND CONCLUSIONS

It is doubtful that the sudden increase of daily lighting following July 5 was the cause of the heat periods of the four females giving birth to kids in December. That question will receive attention in subsequent experiments. It seems much more probable that the continued reduction of daily lighting was becoming increasingly effective in inducing oestrous cycles and heat periods, and the short-time shift to longer days in the pasture failed to inhibit the next heat period in line by reducing the animals' gonadotropic activity below the threshold for induction of heat. It is also possible that a slight shift in the proportions of the hormones concerned may have occurred to bring on such a quick and uniform mating response in the 6 days which appear to have covered the fertile matings of the four reacting animals out of the five used.

In any case, the results of the experiment indicate that reversal of the seasonal cycle of length of daily period of exposure to light of both Toggenburg and Nubian goats leads to partial reversal of the sexual or breeding cycles of the animals. Artificial increase of daily lighting was followed by cessation of heat periods in February instead of in March for experimental animals. Reduced lighting, with temperatures not varying from the normal for the seasons, was accompanied or followed by initiation of heat periods in May and June instead of in September in experimental animals. Even though the signs of heat were neither so intense nor so prolonged as usual, in these experimental animals in May and June, and led to only one mating and no kids, the fact of their appearance is evidence that altered lighting is effective in controlling the oestrous cycles of these animals. The heat periods in July, following return to normal daylight cycles, cannot be open to the above objections, for they led to four pregnancies and production of seven kids.

It is probable that further study may make possible a schedule of controlled lighting which will induce fertile matings at desired intervals in the anoestrous or sterile period of the female goat. In any case the hiatus of births and the reduced milk production consequent to it can be bridged in by this method of controlled lighting. The births in December are almost 2 months before the normal time of first spring births.

SUMMARY

1. Increasing daily light periods from January 25 to April 5—followed by diminishing periods until July 5, while temperatures remained normal for the seasons, with four Toggenburg female goats and one male Toggenburg and one Nubian female—led to cessation of breeding cycles in February instead of March, followed by initiation of breeding cycles in May and June instead of September. Controls retained normal breeding cycles.

2. Matings in July within 10 days of return to normal lighting and continuous pasture with the experimental male produced seven kids from four of the five females used, all born between December 11 and 16, respectively. This is in contrast with first births from controls in February.

3. Two of the four females producing kids were good milkers under these conditions; the other two were light milkers.

4. Results indicate that breeding cycles in goats are controlled by daily periods of light in such a way that short days induce breeding cycles while long days inhibit them. The temperature cycle is not a major factor in environmental control of sexual or breeding cycles in goats.

LITERATURE CITED

BISSONNETTE, T. H. 1936. Sexual photoperiodicity. Quart. Rev. Biol., **11**:371–86.

———. 1938. Experimental control of sexual photoperiodicity in animals and possible application to wild life management. Jour. Wild Life Management, **2**:104–18.

HOOVER, E. E., and HUBBARD, H. F. 1937. Modification of the sexual cycle of trout by control of light. Copeia, 1937, No. 4, pp. 206–10.

MARSHALL, F. H. A. 1936. The Croonian lecture. Sexual periodicity and the causes which determine it. Phil. Trans. Roy. Soc., B, **226**:423–56.

———. 1937. On the change over in the oestrous cycle in animals after transference across the Equator, with further observations on the incidence of the breeding seasons and the factors controlling sexual periodicity. Proc. Roy. Soc., B, **122**:413–28.

WELLS, L. J., and ZALESKY, M. 1940. Effects of low environmental temperature on the reproductive organs of male mammals with annual aspermia. Amer. Jour. Anat., **66**:429–47.

MAXIMAL DENSITY AND PHASES OF DEATH IN POPULATIONS OF GLAUCOMA PIRIFORMIS

(Six figures)

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THE growth of protozoan populations in pure cultures has not been investigated extensively, although the observations of Phelps (1935, 1936) and Loefer (1936) have indicated that growth of ciliates is not markedly different from that of bacteria. In fact, Phelps (1935) has described several of Buchanan's phases of growth—initial stationary, lag, logarithmic growth, negative growth acceleration, and maximal stationary phase—in cultures of *Glaucoma piriformis*. However, the observations of Phelps were limited to the first few days of incubation, and complete histories of populations are known for only one ciliate—*Paramecium bursaria* (Loefer, 1936). Hence, present knowledge of the later phases in growth of protozoan populations is very limited. In view of this situation, the writers have attempted to trace growth of *G. piriformis*, with particular attention to the phases following the maximal stationary phase in which maximal density of population is reached.

MATERIAL AND METHODS

The bacteria-free stock strain of *G. piriformis* and the experimental cultures were grown in the following solution: casein-peptone (Difco Tryptone), 10.0 gm.; KH_2PO_4, 1.0 gm.; distilled water, 1.0 liter. No adjustment of pH was necessary, and the initial pH in all series was 6.3 (±0.1). The medium was tubed in 9.0-cc. amounts for each series and then sterilized in the autoclave for 20 minutes at 122° C. Stock cultures for inoculation of the various series were grown in flasks of this medium.

Two different preparations of casein-peptone (Tryptone) were used. The two samples differed in date of purchase, in color, and in the populations which they supported. The older preparation was relatively light in color and supported maximal densities of approximately 150,000 ciliates per cubic centimeter. The medium containing this peptone is referred to below as Medium A and was used in Series I–III. The second sample, purchased about a year later than the other, was darker in color and supported much lower populations, as described in Series IV, V, and IX. The medium containing the darker peptone is referred to as Medium B. In Series VI–VIII, the two types of peptone were mixed, as specified below.

Each tube in the experimental series received a 0.5-cc. inoculum from a young stock culture. After inoculation one tube was used for determination of initial pH, and several were fixed for the initial counts (number of ciliates per cubic centimeter). During incubation several tubes were fixed at each of the intervals indicated in the separate series; the counts from these tubes, after correction for evaporation, trace growth of the various populations. Our method of counting has been described previously (Hall, Johnson, and Loefer, 1935).

All series were incubated in darkness. Series I was maintained in an incubator at 24° C. for 13 days; at this point, failure of a power line necessitated transfer to room temperature (observed range, 24°–26°). Series III was incubated at room temperature at a range of 22°–26°. The remaining series were incubated at 25° (±0.1).

GROWTH IN TWO DIFFERENT MEDIA

Series I.—This series, inoculated from a 42-hour stock culture, showed an initial count of 5,425 ciliates per cubic centimeter. The maximal stationary phase, in which the population approximated 154,000 per cubic centimeter, was reached during the fourth day and was maintained for less than 48 hours (Fig. 1). Thereafter, the population showed a rapid decline to approximately 60,000 per cubic centimeter on the tenth day. During the next 25 days there was a gradual decline to approximately 40,000 ciliates per cubic centimeter. At this point only a few tube cultures remained, and these were saved for subsequent observations and viability tests. Active ciliates were still present in the cultures at the end of 8 months.

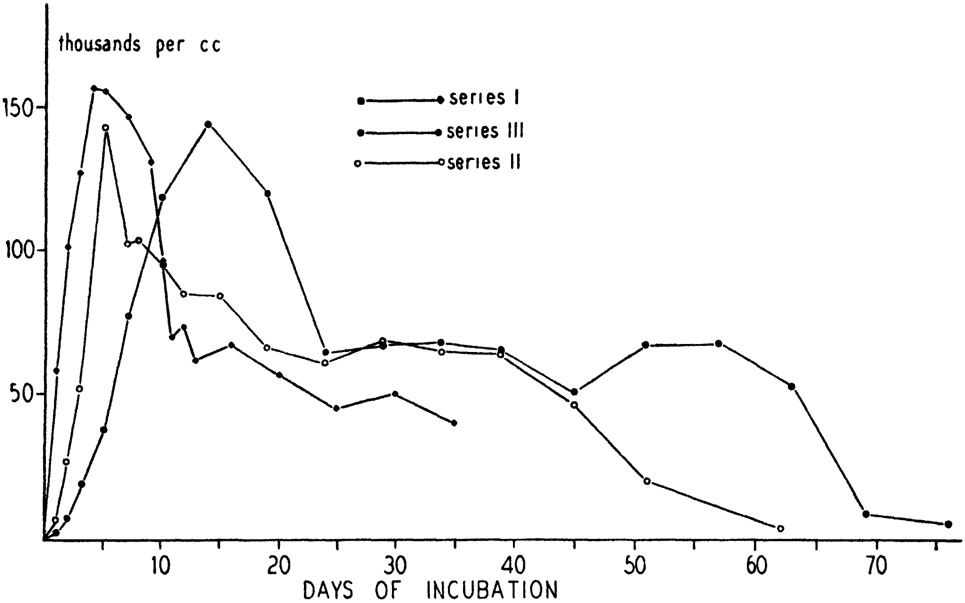

FIG. 1.—Growth of populations in Series I–III, decribed in point-to-point curves. Counts were discontinued at the last point indicated in each series. However, *G. piriformis* continued to live for 2–7 months longer, as noted in the text.

Series II.—A 22-hour stock culture was used in inoculating this series, and the initial count was 490. Growth was traced for approximately 2 months (Fig. 1). The maximal density of population (maximal stationary phase) was reached about the fifth day of incubation. By the seventh day a rapid decrease in population was well under way, and this decline continued, although at a decreasing rate, for more than 2 weeks. This phase was followed by a stationary phase of approximately the same duration, in which the population remained practically constant. After about 40 days of incubation, another phase of death was initiated, and the population was reduced to less than 25,000 per cubic centimeter by the fifty-first day. Subsequently, the rate of death decreased, although there was a further drop in density to approximately 3,000 per cubic centemeter at the end of 62 days. Residual cultures still contained active ciliates at the end of 4 months, when observations were discontinued.

Series III.—In this series, inoculated from a 20-hour stock culture, the initial count was 180. The maximal density of population (Fig. 1) was reached later than in either Series I or Series II, and the subsequent decline in population was somewhat less rapid. The secondary stationary phase following the first phase of death lasted approximately 30 days and was succeeded by a second phase of death similar to that observed in Series II. Counting was discontinued after 76 days, and the few remaining cultures were preserved for later observations. Active ciliates were still present in small numbers after 8.5 months of incubation.

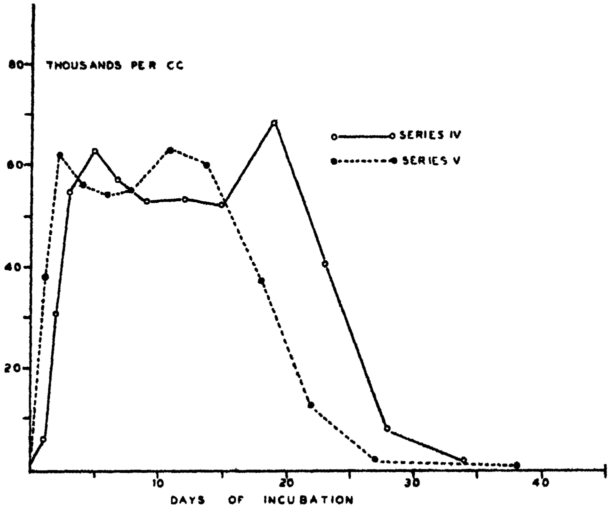

FIG. 2.—Growth of *G. piriformis* in Series IV and V (Medium B). Counts were discontinued at the last point in each series, although residual cultures still contained living ciliates at the end of 3.5 months.

Series IV and V.—In these two series Medium B was substituted for Medium A. Otherwise the experimental procedure was unchanged. Series IV was inoculated from a 28-hour culture, and Series V from a 51-hour stock culture. The respective initial counts were 800 and 3,125 ciliates per cubic centimeter.

In Series IV (Fig. 2) the population had increased to a density of approximately 63,000 per cubic centimeter on the fifth day of incubation. Thereafter, the density remained within the range 53,000–68,000 per cubic centimeter for approximately 2 weeks. This phase was followed by a major phase of death, during which the population dropped to about 8,000 per cubic centimeter within a period of 9 days. During the next 7 days, however, the decline was less rapid; furthermore, cultures incubated for later examination still showed active ciliates after 3.5 months.

Growth in Series V (Fig. 2) was similar to that in Series IV, although the phase of maximal density appeared earlier and passed earlier than in the preceding series. Just as in Series IV, residual cultures still showed active ciliates at the end of 3.5 months.

The observations on Series I–III suggest that the normal growth curve of *G. piriformis* in Medium A approximates that described in Figure 3. Within the first 2 weeks of incubation the population reaches a maximal stationary phase (or phase of maximal density), which is maintained for not more than 2–4 days. This phase, in which the population approximates 150,000 per cubic centimeter, is followed by a phase of death lasting for a week or more. Subsequently, there is a secondary stationary phase in which the population remains almost constant or else declines gradually over a period of 2–4 weeks. A second phase of death follows, and this is succeeded by a tertiary stationary phase in which a slowly decreasing population may be maintained at a low level for several months.

The type of growth curve in Medium B (Fig. 3) differs in several respects from that characteristic of *G. piriformis* in Medium A. The phase of maximal density in Series IV and V shows a population of less than half the density observed in Series I–III and in this respect is comparable to the secondary stationary phase mentioned above. After the peak is first reached in Series IV and V, the population remains fairly constant for 10 days or more, variations falling within the range of 10–20 per cent. This phase of maximal density is succeeded by a phase of death, so that at the end of 30 days or more the population is reduced to the general level observed in Series II and III after approximately 2 months of incubation. Subsequently, the population may be maintained at a low level for at least an additional 2 or 3 months.

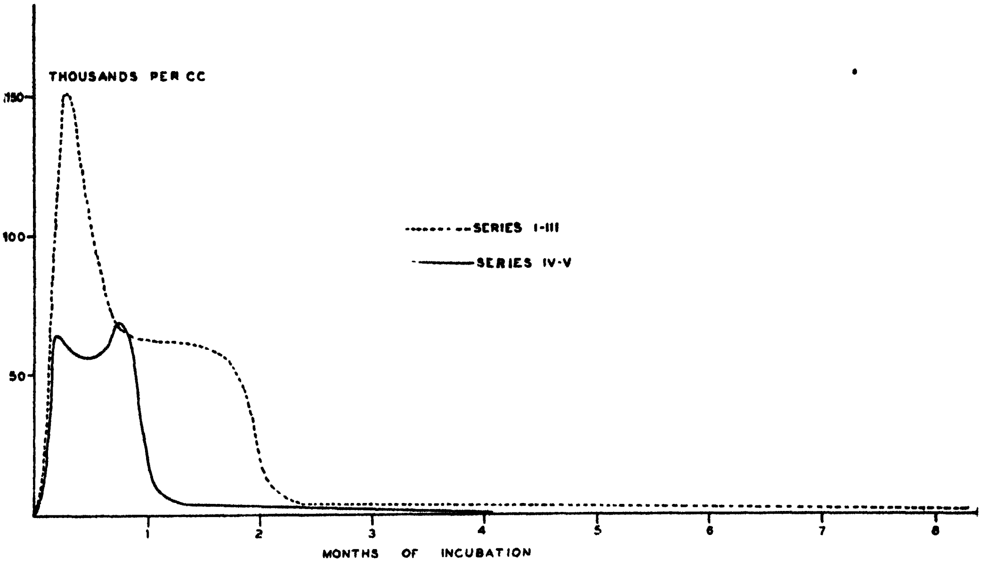

FIG. 3.—Generalized growth curves for *G. piriformis* in two different peptone media. The curve with the higher peak is characteristic of Medium A (Sers. I–III); that with the lower peak, of Medium B (Sers. IV and V).

DIVISION RATES IN SERIES I–V

Calculated division rates (generations per day) for Series I–V afford no explanation for the differences in the growth curves characteristic of the two media. On the first day of incubation (Fig. 4) the number of generations ranged from 2.9 to 3.6, but series in both media are represented at the two extremes. After the first day the decrease in division rate was rapid in every case. Of the five series, I and V are closely comparable with respect to generation times, although the two series were maintained in different media. Likewise, Series II, III, and IV are somewhat similar for the first 3 days of incubation; Medium B was used in Series IV and Medium A in the other series. A comparison of initial counts shows that Series I (5,425 ciliates per cubic centimeter) and Series V (3,125) are fairly comparable; Series II (490), Series III (180), and Series IV (800) may be grouped together on the same basis. The generation curve for Series III, with an initial density of 180 ciliates per cubic centimeter, shows a more gradual negative slope than that of any other series. It thus appears that the division rate for the first 2 or 3 days is correlated with the initial density of population rather than with the type of peptone used in the medium.

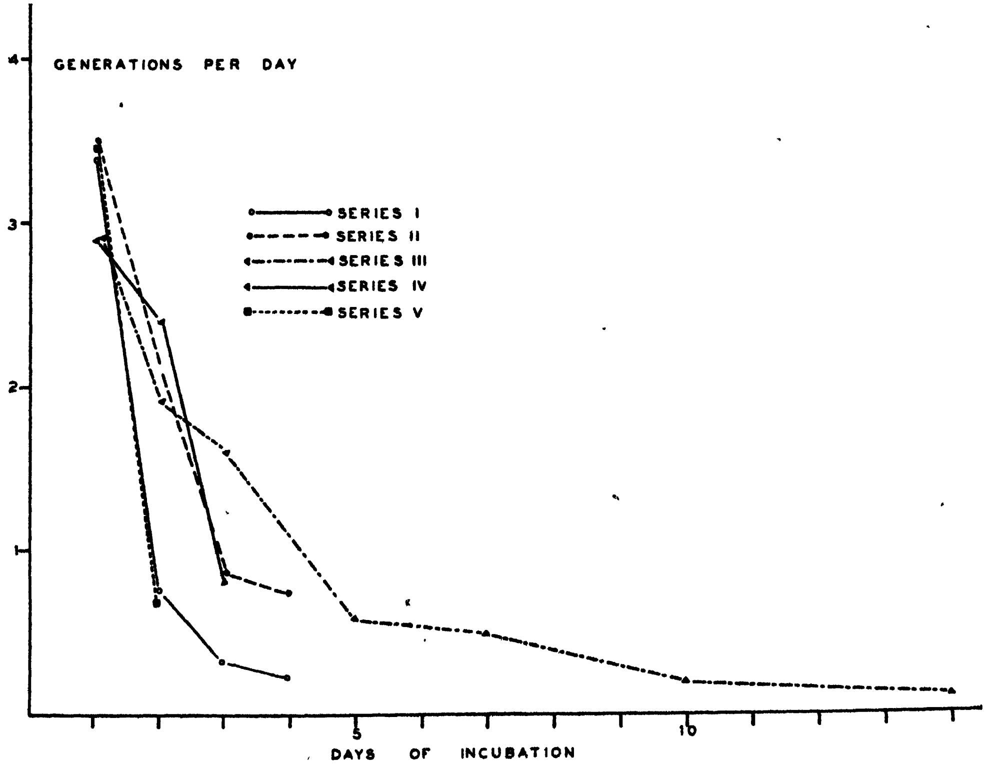

FIG. 4.—Number of generations per day in Series I–V. The final point in each curve coincides with that at which maximal density of population is attained.

GROWTH IN MIXTURES OF TWO PEPTONES

The striking differences between the populations supported by the two peptone media suggested the following observations on mixtures of the two peptones.

In Series VI our two samples of casein-peptone were mixed in the proportion of four parts light peptone to five parts dark peptone. Each tube was inoculated from a 22-hour stock culture in Medium B, and the initial count was 116 per cubic centimeter. The resulting growth curve (Fig. 5) is intermediate between the type observed in Series I–III and that described for Series IV and V, and the maximal density (112,000 ciliates per cubic centimeter) is proportionately intermediate. Active ciliates were still present in residual cultures after 3 months.

Series VII was similar to Series VI, except that the solution of light peptone (Medium A) had been stored in a flask for approximately 7 weeks after sterilization. This was mixed with a fresh solution of dark peptone (Medium B) in the same proportion as in Series VI. The series was inoculated from a 28-hour stock culture in Medium B, and the initial count was 800 per cubic centimeter. Correlated with the higher initial density, there was a sharper rise to the phase of maximal density than was observed in Series VI, and the major phase of death was initiated at least a week earlier. Residual cultures contained living ciliates at the end of 3.5 months. Like Series VI, Series VII presents a growth curve of the intermediate type and thus demonstrates that, whatever the factor responsible for a high maximal density may be, this factor is still operative after sterile Medium A has been stored for several weeks.

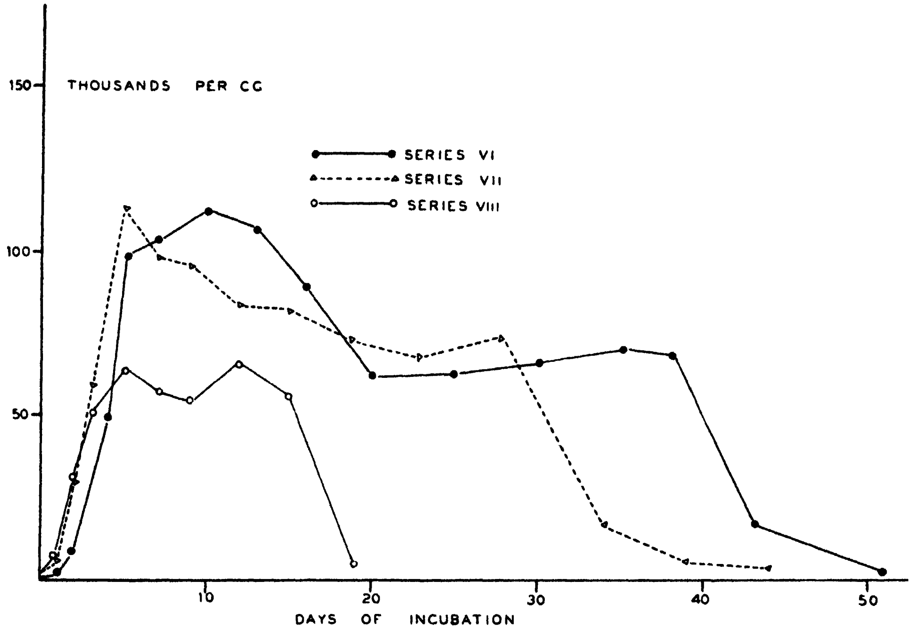

FIG. 5.—Growth in mixtures of the two peptones, Series VI-VIII

Series VIII represents an attempt to determine whether or not the factor favoring dense populations is still detectable in material from old cultures of *G. piriformis* in Medium A. The medium contained four parts of fresh Medium B and five parts of a filtrate from a 50-day culture in Medium A. The series was started from a 28-hour stock culture in Medium B, and the initial count was 800. For the first few days of incubation, growth of the population (Fig. 5) was similar to that in Series VII, in which the initial density was also 800 per cubic centimeter. In Series VIII, however, the population reached a peak of approximately 60,000 per cubic centimeter, just as in Series IV and V—a density little more than half as great as that observed in Series VI and VII. Thus the effect of adding the culture filtrate was the same as if no light peptone at all had been added to the medium.

EFFECTS OF ADDED THIAMINE (VITAMIN B_1)

The results obtained in Series I–VIII suggested that one of our preparations of casein-peptone contains a factor which, in appropriate concentrations, supports a large population for a short period and maintains a moderately dense population for a month or more thereafter. When the concentration of this substance is reduced, as in a mixture of the two peptones, the early rise in population is decreased proportionately (Fig. 5) and the secondary stationary phase is shortened. This substance apparently does not accelerate fission; instead, its effect is to prolong the period during which the population continued to increase, and this effect is proportional to the concentration of the unknown substance. Whatever the factor may be, it apparently is destroyed or inactivated by the ciliates, since it is either absent or reduced to an ineffective concentration in old cultures (Ser. VIII). The apparent properties of this substance suggested that it might be a growth factor present in the casein from which our peptones have been derived. Since thiamine (vitamin B_1) is known to be essential to growth of several ciliates, it seemed

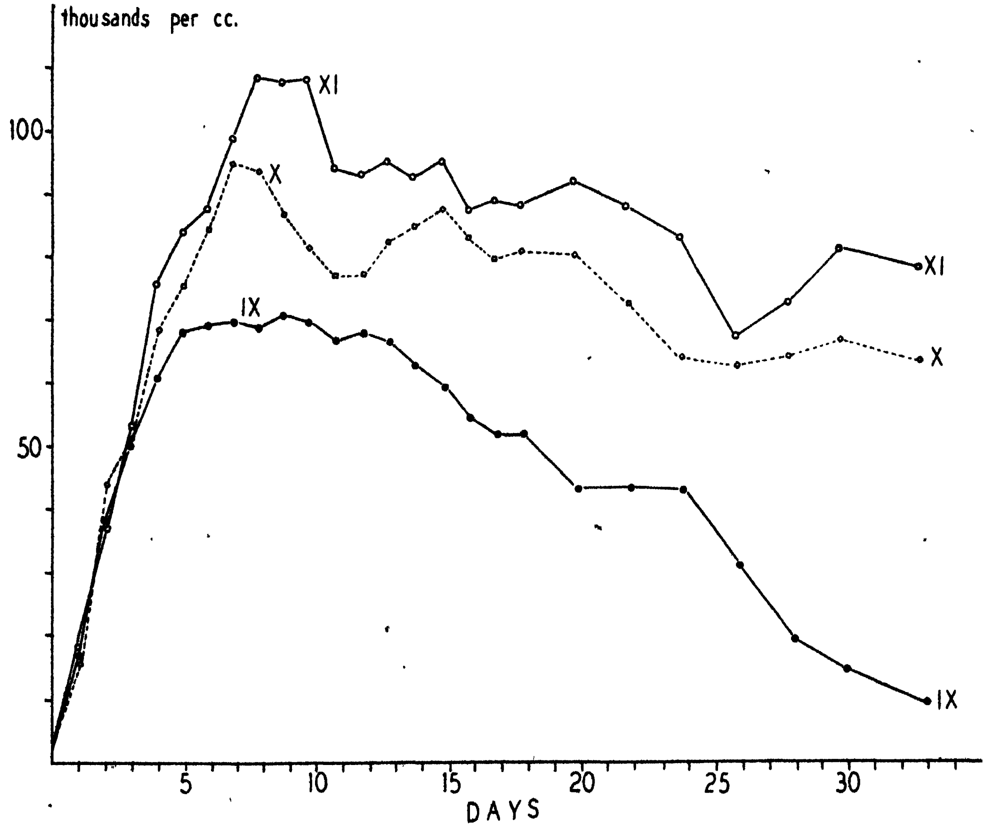

FIG. 6.—Growth of *G. piriformis* in Medium B with and without added thiamine. Series IX, control, Medium B; Series X, Medium B with thiamine, 1×10^{-7} gm. per cubic centimeter; Series XI, Medium B with thiamine, 1×10^{-6} gm. per cubic centimeter.

possible that our Medium B might contain less than an optimal concentration of this growth factor. Hence, this substance was added to Medium B in Series X and XI, as described below.

Series IX–XI.—Medium B was used as the control medium in Series IX; in Series X thiamine was added to this medium in a concentration of 1×10^{-7} gm. per cubic centimeter, while ten times as much thiamine was added in Series XI. All three series were inoculated from a 6-day culture of *G. piriformis* in Medium B, and the initial count was 1,832 ciliates per cubic centimeter. The three populations were traced for a period of 33 days.

In the controls (Ser. IX) the phase of maximal density was reached on the fifth day (Fig. 6), with a population of about 70,000 ciliates per cubic centimeter. With added thiamine (1×10^{-7} gm. per cubic centimeter) in Series X, the population approached 95,000 on the seventh day of incubation. In Series XI, with a higher concentration of thiamine (1×10^{-6}), the population reached a density of approximately 110,000 per cubic centimeter on the eighth day. In Series IX the population remained practically constant for about a week and then began a fairly steady decline. In Series X the population ranged between 78,000 and 88,000 per cubic centimeter during the second and third weeks of incubation and then dropped to a density of approximately 65,000 which was maintained for the last 10 days of incubation. In Series XI the population dropped slightly after the tenth day and then varied between 88,000 and 95,000 per cubic centimeter through the third week; during the last 10 days of incubation the number of ciliates ranged from 70,000 to 80,000, approximately.

The results demonstrate that the available concentration of thiamine definitely affects the density of population during the maximal stationary phase and the secondary stationary phase and also influences the time at which the major phase of death begins.

DISCUSSION

The present observations represent the first attempts to trace the later history of populations in cultures of *G. piriformis*. The early phases of growth in this species have been described by Phelps (1935) and hence have not been considered in detail. Our findings demonstrate phases not previously described for protozoan populations, and they also raise certain questions which cannot yet be answered completely.

As described for bacteria and yeasts, the maximal stationary phase merges into a "phase of accelerated death," and the latter leads into a phase characterized by a more or less constant death rate. In *G. piriformis*, on the other hand, the phases of death are more complicated. In one of our media the phase of maximal density is followed by a phase of death in which the population is reduced by more than one-half. This rapid decline is succeeded by a secondary stationary phase, in which the population may show a gradual decline or may remain practically constant for several weeks. This second stationary phase is followed by a major phase of death, in which the population drops to a low level within a period of approximately 1 week. Finally, the population enters a phase suggesting a tertiary stationary phase, in which the density decreases very slowly and is maintained at a low level for several months. The exact length of this final phase has not been determined, although living ciliates were still present in cultures of different series when observations were discontinued after 4.0–8.5 months of incubation.

The behavior of populations in a second preparation of casein-peptone was quite different, although the division rate of young cultures was apparently the same as for cultures in the other medium. Growth of the population in this second medium ceases at a density approximating 60,000–70,000 ciliates per cubic centimeter, and the population shows little change in density for the next week or two. Next there occurs a major phase of death in which the population is reduced to a low level. These phases are completed in about a month—approximately half the time required in the other medium. The final phase, however, is much the same in the two cases, since populations of low density have already persisted for over 3 months in the second peptone medium.

The results obtained in Series I–VIII indicate that one of our peptones (Medium A) contains a factor which does not accelerate fission appreciably but does increase the length of the period during which growth of a ciliate population continues. The substance may be present also in the second type of peptone (Medium B), but only in a concentration which supports a population of moderate density for a relatively short time. The effect exerted by the substance is proportional to its concentration, and the substance itself apparently is inactivated or destroyed by the ciliates.

A preliminary attempt to identify this postulated substance as thiamine is described in Series IX–XI. The results indicate that an adequate concentration of thiamine is essential to the secondary stationary phase observed in Series I–III. Series IX, in Medium B, showed a fairly steady decline in density of population after the second week of incubation; in Series X and XI, on the other hand, the behavior of the populations resembled that in the secondary stationary phase of Series I–III. In fact, the density of population in Series XI was appreciably higher than that in Series I–III during this phase. Hence it may be assumed that the concentration of thiamine used in Series XI was higher than that available in Medium A (Sers. I–III).

Such an assumption implies that thiamine cannot be the only factor controlling the phase of maximal density (approximately 150,000 ciliates per cubic centimeter) in Series I–III. Series X and XI show an early peak (Fig. 6) which is suggestive of the maximal stationary phase observed in Medium A, but the maximal density of population is in each case much lower than that in Series I–III. It seems probable that the effect of higher concentrations of thiamine would be limited, as far as the maximal stationary phase is concerned, since a tenfold increase in thiamine (Ser. XI) raised the population level less than 20 per cent above that observed in Series X. Theoretically, it would be necessary on this basis to increase the concentration of thiamine to the order of 1×10^{-4} gm. per cubic centimeter in order to produce a maximal stationary phase approaching that observed in Series I–III. Since such a concentration of thiamine presumably would exert proportional effects during the secondary stationary phase, the results would not duplicate the growth curves obtained in Series I–III. Hence it seems necessary to conclude that other factors as well as thiamine are probably involved in the appearance of the maximal stationary phase characteristic of Medium A (Sers. I–III).

Other possibilities are now being considered. Preliminary observations (Hall, 1942) indicate that riboflavin (vitamin B_2) is probably essential to growth of *Colpidium campylum* and suggest that nicotinamide may also be an essential growth factor for this ciliate. It is possible that these substances may be equally important in the metabolism of *G. piriformis.* Hence both riboflavin and nicotinamide are being tested for their effects on density of population in cultures of *G. piriformis*. These studies are being combined with observations on the effects of thiamine in concentrations higher than those used in Series X and XI.

In conclusion, however, it may be pointed out that the density of population in cultures of *G. piriformis* is related quantitatively to the concentration of thiamine and that the phases of death also are influenced markedly by the available concentration of this vitamin. Thus, Series X and XI showed populations of approximately 65,000 and 80,000, respectively, after 1 month of incubation, whereas Series IX (Medium B without added thiamine) showed a population of less than 10,000 ciliates per cubic centimeter. In other words, addition of thiamine to Medium B converted the major phase of death, as observed during the third and fourth weeks of incubation (Sers. IV, V, and IX), into a phase resembling the secondary stationary phase noted in Medium A (Sers. I–III).

These results have some bearing on the relation of protozoan waste products to the behavior of populations. The steady decline in Series IX after the second week of incubation, considered separately, might have been attributed to the accumulation of toxic wastes. However, the addition of thiamine in Series X and XI definitely postponed this major phase of death, although the populations reached higher levels than in Series IX and more waste products should have been produced within a shorter period. In unpublished observations on *C. campylum* a solution of thiamine was added to cultures after the maximal stationary phase was reached, while controls received the same quantity of sterile distilled water. The effect of thiamine added at this time was much the same as that observed in Series XI, in which the thiamine was added before inoculation of the cultures. Accordingly, it seems obvious that death of the majority of ciliates in Medium B (Sers. IV, V, and IX) within the first 4 weeks of incubation is dependent upon a deficiency of thiamine.

LITERATURE CITED

HALL, R. P. 1942. Incomplete proteins as nitrogen sources and their relation to vitamin requirements in *Colpidium campylum*. Physiol. Zoöl. (In press.)

HALL, R. P.; JOHNSON, D. F.; and LOEFER, J. B. 1935. A method for counting Protozoa in the measurement of growth under experimental conditions. Trans. Amer. Micr. Soc., **54**:298–300.

LOEFER, J. B. 1936. Bacteria-free cultures of *Paramecium bursaria* and concentration of the medium as a factor in growth. Jour. Exper. Zoöl., **72**:387–407.

PHELPS, A. 1935. Growth of Protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and of the amount of the inoculum. Jour. Exper. Zoöl., **70**:109–30.

———. 1936. Growth of Protozoa in pure culture. II. Effect upon the growth curve of different concentrations of nutrient materials. *Ibid.*, **72**:479–96.

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STUDIES IN POPULATION PHYSIOLOGY. X. INTERSPECIFIC COMPETITION IN POPULATIONS OF GRANARY BEETLES[1]

(Fourteen figures)

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INTRODUCTION

POPULATIONS can be studied experimentally by using either a single-species or a mixed-species culture. In the former case attention centers on the analysis of a comparatively simple type of biological association. There is an attempt to assay as many mass relations between the organisms and their environment as possible. These studies are "intraspecific" and are typified by the investigations of Johnson (1933) and Reich (1936) on microörganisms; of Pearl (1932) and Bodenheimer (1938) on *Drosophila* populations; of Chapman (1928) and Park (1941) on *Tribolium;* and of Allee and Evans (1937) on *Arbacia punctulata.* The mixed-species or "interspecific" studies are concerned primarily with analysis of those competitive (or co-operative) relations established within a population when several species with essentially similar ecological requirements live together. On an experimental basis this analysis demands (1) that an empirical description of the growth and trend of the entire population shall be worked out and (2) that the factors associated with or causing the observed trends shall be understood. The two most-considered interactions between species have been predator-prey relationships and the competition of two species for food and *Lebensraum.* This interest was focused by the theoretical writings of Lotka (1934), Volterra (1928), and Nicholson (1933); by the obvious implications of interspecific behavior for evolution and speciation; and by the observations of ecologists on competitive phenomena in natural populations. However, with a few exceptions, there has been little attempt to analyze competition or the "struggle for existence" experimentally. The notable exceptions are the work of Gause (1934) on microörganisms; of Gause, Smaragdova, and Witt (1936) and Smaragdova (1936) on the mites *Cheyletus* (predator) and *Aleuroglyphus* (prey); of Omori (1939) on two species of *Cimex;* and of Vladimerova and Smirnov (1938) on the competition between two genera of flies (*Musca* and *Phormia*) for food.

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In this paper another experimental analysis of interspecific competition will be presented. This is a long-time project. Although the data discussed here are based on more than two years' continuous research, they are essentially descriptive in character. They are suggestive in that new lines of attack are opened for future experimentation. This is not an apology. In population work it is particularly desirable to build a picture of growth trends based on the entire population before proceeding to detailed analyses of causal factors.

MATERIALS AND METHODS

In an adequate interspecific population study the following are important technical desiderata: first, it must be feasible to culture all species in the same medium in a restricted space; second, the medium should be reproducible both as to quality and as to quantity; third, the husbandry of each species must be relatively simple; fourth, a method of taking a census must exist (this method cannot be so drastic that it injures the organisms, and it must yield accurate results); and, fifth, it must be possible to reconstitute the population after each census and to maintain the medium and the control cultures as long as the experiment warrants. For the present experiments three species of granary beetles have been selected. They appear to fulfil a number of the requirements set forth above. The beetles are *Tribolium confusum*, the flour beetle, *Gnathoceros cornutus*, the broad-horned flour beetle, and *Trogoderma versicolor*. The first two are Tenebrionids; the latter a Dermestid. All three species came from closely inbred stocks, the *Tribolium* from the authors' laboratory and the *Trogoderma* and *Gnathoceros* from the Stored Products Research Laboratory of Slough, England.

Medium.—Some difficulty was encountered in devising a medium that could be sifted for census-taking and, at the same time, that would be suitable nutritionally for all three species of beetles. *Tribolium* lives well in a finely milled flour (for husbandry see Park, 1934). This is ideal since the flour is readily sifted and, with a minimum of effort, eggs, larvae, pupae, and imagoes can be collected for counting. *Gnathoceros* and *Trogoderma* require coarse as well as fine particles in their medium. This complicates the census since it is tedious to segregate the stages when the larger particles are present. However, as there was no way to avoid this difficulty a medium of the following composition was made up:

70 parts (by weight) of sifted whole-wheat flour
28 rolled oats
2 sifted, dry yeast

The flour ("Lenfesty" whole wheat) and the yeast (Fleischmann's dry brewer's yeast, *Saccharomyces cerevisiae*) are sifted through a silk bolting cloth with 82 meshes to the inch. This sieve retains all metamorphic stages of all three species. The coarser middlings and bran are discarded, and the fine flour (with the yeast) is used. The rolled oats ("Quaker" brand) are sifted through a coarse bolting cloth (17 meshes to the inch) to remove broken flakes and dust. Then flour, yeast, and oats are added together and gently but diligently stirred until the medium becomes a homogeneous mixture consisting of a finely milled component (flour and yeast) smaller than any of the beetles' stages and a coarse component (oat flakes) larger than any of the beetles' stages.

Census procedure.—In making counts it is feasible to sift the medium through a number of bolting-cloth sieves of different-sized mesh in order to segregate the beetles and their metamorphic stages according to size. This procedure facilitates counting and can be outlined as follows in chronological sequence:

1. The total medium is passed through a coarse bolting-cloth sieve (17 meshes to the inch). This removes from the culture the larger flakes as well as imagoes, pupae, and late instar larvae. These flakes are set aside for later examination.
2. The residue is sifted through a fine sieve (82 meshes to the inch). This takes out the major part of the medium as a powder from which all remaining beetle stages (eggs, early and middle instar larvae) have been removed and retained by the sieve. The powder is discarded.
3. The remaining medium is sifted through a sieve (30 meshes to the inch) and the larvae (middle instar) thus collected and set aside.
4. The residue is sifted through a sieve (38 meshes to the inch) and the larvae (early and middle instar) thus collected and set aside.
5. The residue is sifted through a sieve (48 meshes to the inch) and the larvae (early instar) and some eggs thus collected and set aside. The residue contains essentially no eggs or larvae of *Tribolium* and *Gnathoceros*, and the few that are present are removed. *Trogoderma* eggs are elongated cylinders, and some of them pass through the 48-mesh sieve. Unfortunately, these eggs are lost.
6. The sifting is now concluded. The five portions containing medium and beetles are then worked over by hand, and all the beetles' stages except the eggs are segregated for counting. The eggs cannot be counted because they are of the same size as the finest residue and thus cannot be separated. The residue containing eggs is not discarded but returned to the population. Thus the eggs, although not counted, are not lost except in the case of *Trogoderma*, where some eggs pass through the 48-mesh sieve. No residue is discarded until it is examined under the binocular dissecting microscope for larvae or other stages that might have been overlooked.
7. After counting, the following items, depending on the mating in question, are recorded:
   - *a*) For every mating the age of the culture, date of examination, and initials of observers.
   - *b*) For all three species the number of larvae and pupae.
   - *c*) For *Tribolium* the total number of imagoes (these imagoes cannot be sexed).
   - *d*) For *Gnathoceros* and *Trogoderma* the number and sex of the imagoes.
   - *e*) For *Trogoderma* the number and sex of the *dead* imagoes (the significance of this record will appear later).
   - *f*) For all populations any special remarks that might be pertinent.
8. After counting and recording, the new population is started by returning all stages to *fresh* medium and gently redistributing them through their environment.

It is apparent from this description that the census method used in these experiments is a *total* rather than a *sampling* method. This is something of a novelty in population work. It rules out obviously the type of error associated with any sampling technique. At the same time the populations are large enough to avoid the criticism of small numbers.

Environmental control.—Since the experiments are a study in biotic competition, it is necessary to control external environmental factors so far as possible. When this is done, it is legitimate to say that, to a large extent, the results observed are an expression of intraculture dynamics rather than extra-culture differentials. To this end the temperature, light, humidity, quantity and quality of the medium, and handling are controlled rather carefully. All populations are maintained in a dark, thermostat-regulated incubator at a temperature of 29° (±0.5° variation on the average). The relative humidity in the incubators varies from 60 to 70 per cent on the mean, although during July and August it is consistently above this figure. In the winter the humidity is raised by placing trays containing sand in the bottom of the incubator. All populations are kept on the top shelf of the same incubator so that what variation does exist is common for all. The culture containers are wide-mouth bottles, 100 mm. high and 70 mm. in diameter. One hundred grams of medium is placed in each culture bottle. The medium is allowed to come into temperature and moisture equilibrium with the incubator's environment since it is prepared and bottled a week or more before using. Handling is standardized by sifting mechanically and by following the same routine in examination of all cultures. The beetles are handled as gently as possible with brush and spatula.

The experimental design.—The experiments reported in this paper are designed so that (1) there are records of population growth of *Tribolium*, *Gnathoceros*, and *Trogoderma* grown as pure cultures (single species); these constitute controls; (2) there are records of population growth when three species are cultured in all combinations of twos with both species initially seeded in equal imaginal density; and (3) there are records of population growth when all three species are cultured in all combinations of twos with one species initially seeded in a higher imaginal density than its competing mate. The experiments are set up as follows:

Mating	Code Letter
44 *Tribolium* males × 44 *Tribolium* females	A
44 *Gnathoceros* males × 44 *Gnathoceros* females	B
44 *Trogoderma* males × 44 *Trogoderma* females	C
22 *Tribolium* males & 22 *Tribolium* females × 22 *Gnathoceros* males & 22 *Gnathoceros* females	D
22 *Tribolium* males & 22 *Tribolium* females × 22 *Trogoderma* males & 22 *Trogoderma* females	E
22 *Gnathoceros* males & 22 *Gnathoceros* females × 22 *Trogoderma* males & 22 *Trogoderma* females	F
38 *Tribolium* males & 38 *Tribolium* females × 6 *Gnathoceros* males & 6 *Gnathoceros* females	G
38 *Tribolium* males & 38 *Tribolium* females × 6 *Trogoderma* males & 6 *Trogoderma* females	H
38 *Gnathoceros* males & 38 *Gnathoceros* females × 6 *Tribolium* males & 6 *Tribolium* females	I
38 *Gnathoceros* males & 38 *Gnathoceros* females × 6 *Trogoderma* males & 6 *Trogoderma* females	J
38 *Trogoderma* males & 38 *Trogoderma* females × 6 *Tribolium* males & 6 *Tribolium* females	K
38 *Trogoderma* males & 38 *Trogoderma* females × 6 *Gnathoceros* males & 6 *Gnathoceros* females	L
15 *Tribolium* males & 15 *Tribolium* females × 15 *Gnathoceros* males & 15 *Gnathoceros* females × 15 *Trogoderma* males & 15 *Trogoderma* females	M

In the present paper a particular mating will be referred to by its code letter. When it is necessary to indicate a replication, a superscript will be used. Thus F^2 designates the second culture started with *Gnathoceros* and *Trogoderma* in equal density.

The experimental procedure can be summarized as follows: (1) All cultures are maintained under the same environmental conditions; (2) all cultures are started with 88 young imagoes in 100 gm. of medium (except the M-mating which is a special case); (3) all cultures are examined every 30 days. At that time records of numbers are taken, and the beetles are returned to their appropriate bottle containing fresh medium. Conditioning of the flour (see Park, 1941, and Park and Woollcott, 1937) thus is kept purposely from being an important variable in these matings.

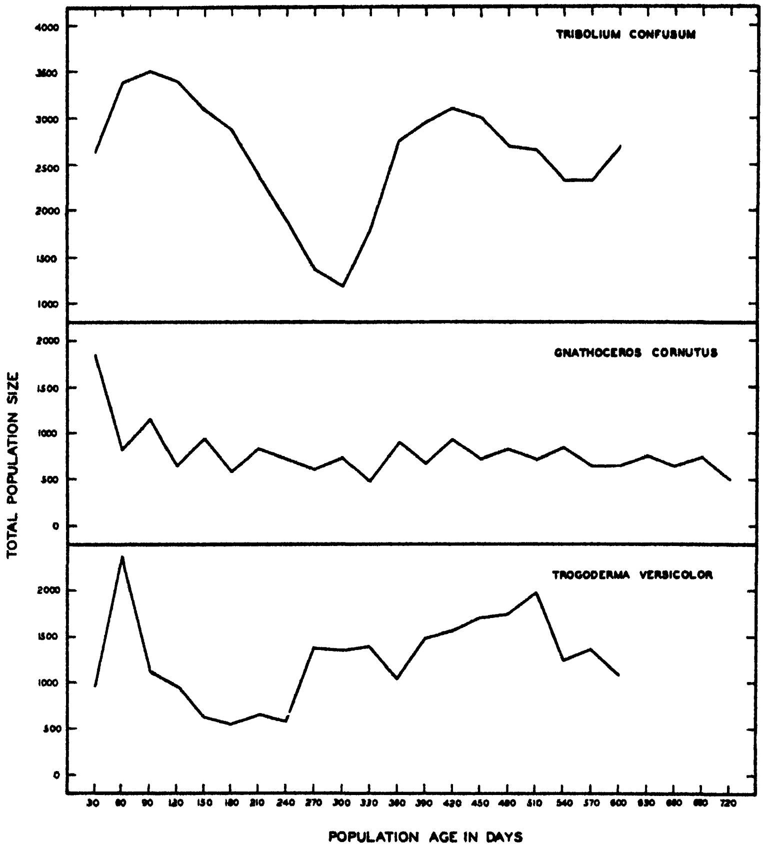

FIG. 1.—Population growth of control cultures of *Tribolium confusum*, *Gnathoceros cornutus*, and *Trogoderma versicolor*. Curves based on means.

In the following discussion we shall consider, first, the growth of the control or single-species populations; second, the competition in the mixed-species cultures and its end effects; and, third, the biological implications of these data.

TABLE 1*

COMPOSITION AND TOTAL SIZE OF *Tribolium* CONTROLS RELATIVE TO HIGH AND LOW DENSITIES

AGE INTERVAL (DAYS)	COMPOSITION OF TOTAL POPULATION IN PERCENTAGES									TOTAL POPULATION (LARVAE, PUPAE, AND IMAGOES)			
	Larvae			Pupae			Imagoes						
	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	Mean	S.D.	C.V.	*n*
30–180 (1st high-density period)	61.1±1.50	13.4±1.06	21.9	5.3±1.02	9.1±0.72	171.7	33.6±2.11	18.8±1.49	55.9	3146.30±74.8	665.8±52.0	21.2	36
210–330 (1st low-density period)	67.1±1.19	9.7±0.84	14.4	4.2±0.29	2.4±0.20	57.1	28.7±1.26	10.3±0.89	35.9	1686.56±72.0	640.6±50.0	37.9	30
360–510 (2d high-density period)	70.3±0.57	5.1±0.40	7.2	7.1±0.50	4.5±0.35	63.4	22.6±0.51	4.6±0.36	20.4	2861.40±61.3	545.2±43.3	19.0	36
540–600 (2d low-density period)	63.5±1.27	8.0±0.89	12.6	12.1±0.76	4.8±0.53	39.7	24.4±0.84	5.3±0.59	21.7	2459.61±108.0	680.9±76.5	27.7	18
0–600 (total period)	65.5±0.73	11.8±0.52	18.0	7.2±0.36	5.8±0.25	80.5	27.3±0.83	13.6±0.59	49.8	2538.46±61.4	997.5±43.4	39.3	120

* The sampling parameter used throughout this paper is the probable error.

GROWTH OF SINGLE-SPECIES POPULATIONS

Tribolium confusum.—The data on *Tribolium* population growth are based on census observations made every 30 days over a 600-day span on six different cultures. The growth trend computed as a mean of these records is shown in Figure 1. This figure immediately suggests the interesting point that these *Tribolium* populations, after reaching their asymptote, do not maintain themselves at that place but, instead, begin a slow decline and, after reaching a low point at about 300 days, start to build up again. A second peak is reached around 420 days, which is followed by another but lesser decline. In order to appreciate better the magnitude of this effect the 600 days' observations are divided into four periods as follows: the first high-density period (30–180 days); the first low-density period (210–330 days); the second high-density period (360–510 days); and the second low-density period (540–600 days). For each of these periods the percentage composition[2] of larvae, pupae, and imagoes is calculated, as well as the mean population size. These data appear in Table 1. The following points emerge from study of this table:

TABLE 2

ANALYSIS OF *Tribolium* CONTROLS WITH LOWEST DENSITIES SET EQUAL

Mean Age	A^1	A^2	A^3	A^4	A^5	A^6	Mean	Deviation from Mean Low
85......	4,234	3,208	3,374	4,425	3,649	2,395	3,547	+2,667
115......	3,790	2,897	3,010	3,918	3,195	2,723	3,256	+2,376
145......	3,439	2,998	2,790	3,825	2,822	3,230	3,184	+2,304
175......	3,039	2,840	2,925	3,687	2,556	2,512	2,927	+2,047
205......	2,472	1,824	2,596	3,012	2,163	2,448	2,419	+1,539
235......	1,894	1,837	1,812	2,556	1,704	1,990	1,965	+1,085
265......	1,056	1,073	1,149	1,931	1,029	1,465	1,284	+ 404
Low 295.....	740	734	1,021	850	623	1,314	880	0
325	1,651	2,024	1,243	1,498	1,764	2,216	1,733	+ 853
355......	2,862	3,445	2,520	2,246	3,355	2,734	2,860	+1,980
385......	3,370	3,647	2,890	2,992	3,809	2,561	3,211	+2,331
415......	3,232	2,914	3,456	2,319	2,604	2,138	2,777	+1,897
445......	2,774	3,249	3,494	3,604	2,218	2,663	3,000	+2,120
475	3,094	2,385	3,042	3,467	2,273	2,399	2,777	+1,897
505......	2,655	1,930	2,544	2,647	1,478	2,849	2,350	+1,470

1. The total population size of the first period (3,146 beetles per 100 gm. medium) is about double that of the 210–330-day period (1,686 beetles).
2. While there are suggestions of a second high (2,861 beetles) and a second low (2,459 beetles) at 360–510 days and 540–600 days, respectively, it is obvious that the oscillations are dampened. The relative variation as measured by the coefficient of variability is somewhat higher for the low- than for the high-density periods.

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[2] Throughout this paper the term "composition" will be used in this specific sense, namely, to denote for a single species the percentage composition of the total culture in terms of larvae, pupae, and imagoes.

3. The mean population size attained by *Tribolium* under these conditions is 2,538 beetles per 100 gm. of medium. The significance of this figure will become apparent when it is compared with that for *Gnathoceros* and *Trogoderma* controls.
4. Larvae form the bulk of the population, with imagoes next and pupae last. The larval counts are also the least, and the pupal the most, variable. Although the percentage of larvae, pupae, and imagoes differs at times significantly from one period to another from the mean for the total 600 days, there appears to be no consistent pattern in these deviations as regards total population size. In sum, it cannot be concluded that percentage composition is related closely to total population density.

The data of Table 1 and Figure 1 are based on means computed by 30-day periods. Thus, for example, the mean population size at 300 days is a mean of six separate cultures. To the reader familiar with population phenomena it is obvious that, although 300 days appears as the low in Figure 1, it is not necessarily the low for every one of the six cultures. This fact is dealt with in Table 2, where the lowest density is chosen arbitrarily from each mating irrespective of the age of the culture and 210 days laid off on either side of that point. Table 2 shows the mean low density to occur at the mean age of 295 days. The table also shows how one culture varies from another. It is apparent that all six populations possess an oscillation cycle and that the decline period tends to be more gradual than the growth period. These data are graphed in Figure 2 on a logarithmic ordinate and arithmetic abscissa. This figure gives a good smoothed picture of the form of the oscillation.

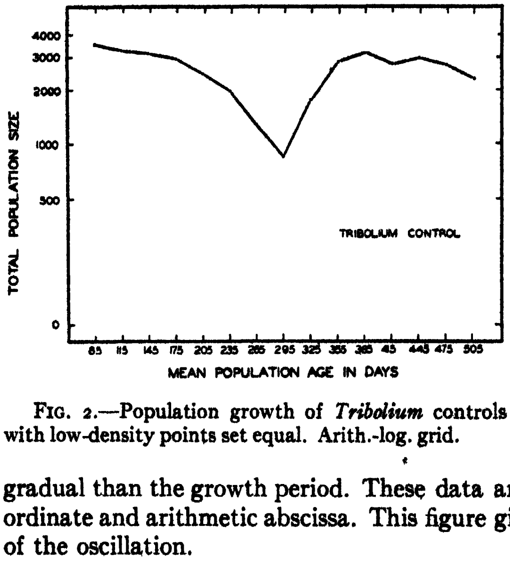

FIG. 2.—Population growth of *Tribolium* controls with low-density points set equal. Arith.-log. grid.

In this paper we shall describe by means of graphs the course of populations treated individually as well as averaged. Although this treatment adds to the bulk of the paper and perhaps may seem irrelevant to the reader, it has two virtues: first, it depicts immediately the interculture variability which in some instances is considerable, and, second, it presents the data in a form that is better suited for possible later analysis by students of theoretical populations. Figure 3 shows the *Tribolium* controls plotted as single cultures. This graph does not change any of the major conclusions just discussed. However, the comparison of the curves is instructive and should be made by the reader.

Bodenheimer (1938) devised a method of representing certain aspects of population dynamics that has some application here. He applied his schema primarily to populations of *Drosophila* and honeybees, although Chapman's (1928) data on *Tribolium* were also used to a lesser extent. Table 3 is a simplification of Bodenheimer's protocol based on the six *Tribolium* control cultures just discussed. In the table Columns A, B, and C are all obvious entries. Column D—the potential increase of the population—represents the maximum number of eggs the population could produce, assuming that there is a consistent oviposition rate, an equal sex ratio, and nothing in the environment to reduce this fecundity. This index is obviously theoretical and arbitrary, but it has a certain interpretative value. Before it can be computed it is necessary to arrive at a standard and empirical expression of fecundity. A good deal of experience with the egg-laying performance of *Tribolium* suggests that a suitable mean figure to use is 90 eggs per female per 30-day period. Thus the potential increase equals

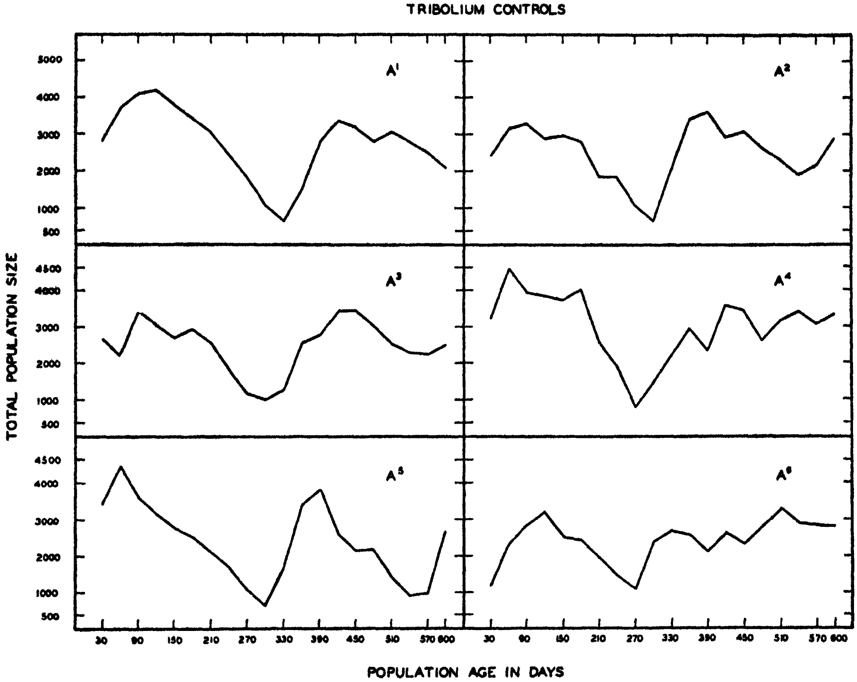

FIG. 3.—Population growth of *Tribolium* controls. Curves based on individual cultures

$$90 \times \tfrac{1}{2}\ \text{(imagoes)}\ .$$

These are the computations appearing in Column D. Column E is an expression of population growth rate per past 30-day period. It is computed as follows:

$$E = \frac{\text{Total population}}{\text{Total population at last 30 days}}\ .$$

This index merely puts in ratio form the change in total population size. If the quotient is greater than 1, the culture is growing; if less than 1, the culture is declining. Column F —the intensity of the struggle for existence—is computed as follows:

$$F = \frac{\text{Potential increase}}{\text{Rate of growth}} = \frac{D}{E}\ .$$

This index is again a quotient and gives in Bodenheimer's opinion a numerical approximation of the stringency of competition. We are not too convinced of the usefulness of this index, in part because we have not followed the logic behind it completely and in part because Bodenheimer does not himself evaluate it critically in his book. We include it in our tables primarily because it has been used by others and may be developed in greater detail in the future.

TABLE 3

Tribolium CONTROLS ARRANGED IN BODENHEIMER'S CLASSIFICATION

(A) Age	(B) Mean Total Population	(C) Mean Number Imagoes	(D) Potential Increase of the Population per Past 30 Days	(E) Rate of Growth	(F) Intensity of the Struggle for Existence
30	2,629.2	90.8	4,086.0	....	....
60	3,387.8	1,551.0	69,795.0	1.28	54,527.3
90	3,500.0	1,524.2	68,589.0	1.03	66,591.3
120	3,398.5	1,382.5	62,208.0	0.97	64,131.9
150	3,092.3	1,226.3	55,179.0	0.91	60,636.3
180	2,870.0	997.0	44,865.0	0.93	48,241.9
210	2,361.3	792.8	35,676.0	0.82	43,507.3
240	1,870.2	567.2	25,524.0	0.79	32,308.9
270	1,368.5	404.0	18,180.0	0.73	24,904.2
300	1,191.3	315.3	14,184.0	0.87	16,303.4
330	1,791.8	331.7	23,922.0	1.50	15,948.0
360	2,754.0	614.8	27,666.0	1.54	17,964.9
390	2,944.2	694.3	31,239.0	1.07	29,195.3
420	3,101.8	699.8	31,491.0	1.05	29,991.4
450	3,009.8	648.3	29,169.0	0.97	30,071.1
480	2,702.0	639.0	28,755.0	0.90	31,950.0
510	2,656.3	572.2	25,749.0	0.95	27,104.2
540	2,320.8	552.3	24,849.0	0.87	28,562.1
570	2,320.7	494.0	22,230.0	0.99	22,454.5
600	2,692.3	677.5	30,483.0	1.16	26,278.4

An examination of Table 3 shows two tendencies. (1) The potential increase starts out low owing to the presence of few imagoes but at 60 days, after a second generation has emerged, rises many fold and maintains a high level during the 30–180-day period. The potential increase drops during the 210–330-day period when the populations are at the bottom of their cycle, and then it rises somewhat as the populations build up toward their second peak. However, it does not rise so high as in the early stages of population growth, since the imagoes do not increase to their earlier level. (2) The intensity of the struggle for existence follows essentially the same pattern. The aggregate competition between beetles is highest during the first period and then drops as the culture wanes. There is some build-up of this component during the third period, but it does not attain the force reached earlier. In the fourth period the intensity drops somewhat but not to the same degree as it did in the second period. On the basis of the observed dampened oscillations this is to be anticipated.

At this point let us ask the question: Why, in a paper dealing with interspecific competition, must so much time be spent on single-species cultures? The answer is, of course, that the latter furnish a control against which the experiments can be tested. In this investigation we must be able to say that the single species live indefinitely as cultured here and that their populations follow a certain growth form. If these two points are known, then deviations in the experiments from the control pattern can be evaluated. For example, if one species that grows well when alone becomes extinct when in association with another species, it can be concluded, other things being equal, that the first species is driven out by the pressure of competition.

Since it has been shown for the present controls that *Tribolium* has a major oscillation during its early growth, it becomes particularly important to have a clear picture of this cycle. The cycle is a property of *Tribolium* and is not due to association with *Gnathoceros* and *Trogoderma*. Nevertheless, it persists when *Tribolium* is living with other species and, as we shall see, frequently is of considerable importance in shaping the total population trend. As to the cause of the oscillations, more experimentation is needed before definite conclusions can be drawn. Some work along this line is in progress. There are some tentative data suggesting that infection of the beetles with a sporozoan, *Adelina* (see Park, 1934), may be responsible. In short, the cycle may represent a pandemic type of phenomenon. Speculation on this point must await addi-

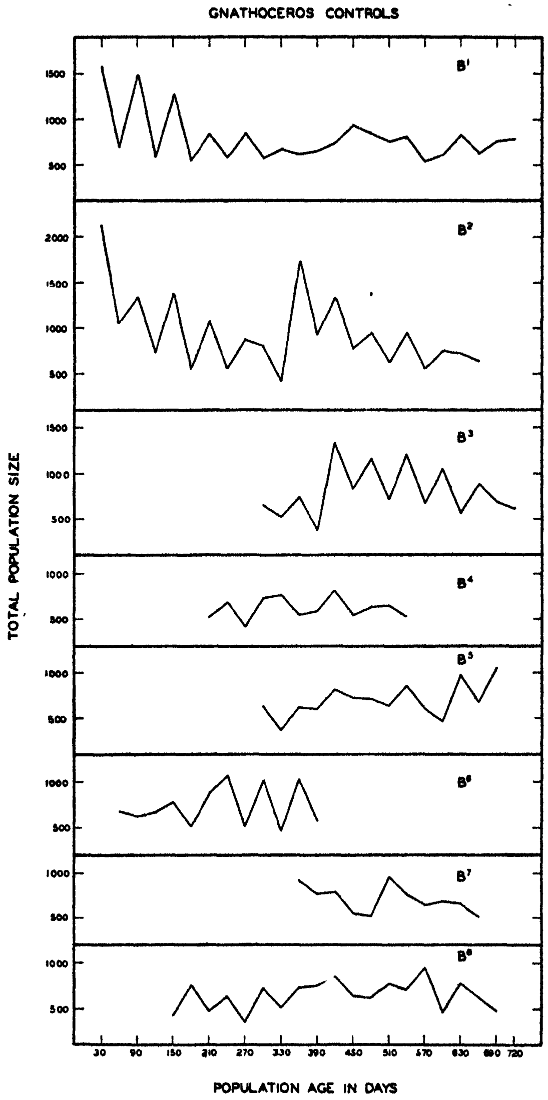

FIG. 4.—Population growth of *Gnathoceros* controls. Curves based on individual cultures. B^3 through B^8 have served as interspecific experimentals during their early history and are used as controls after their competitor becomes extinct.

tional evidence. Chapman (1933) has carried this analysis further than anyone else, and he is of the opinion that oscillations in *Tribolium* populations can be caused by disease infection.

Gnathoceros cornutus.—The data on *Gnathoceros* population growth are based on census observations made every 30 days over a 720-day span on eight different cultures[3] The growth trend computed as a mean of these records is shown in Figure 1, the individual curves in Figure 4, and the data on percentage composition and total population size are summarized in Table 4. The graphs show an entirely different pattern for *Gnathoceros* than for *Tribolium* populations. The former curves are small but less variable and give no evidence of major oscillations. There are minor waves in the curve that suggest, with certain exceptions, that a large census tends to be followed the next 30 days by a smaller census. This "high-low" feature has not been analyzed. It may represent merely a life-cycle phenomenon, i.e., a wave of emergences and subsequent reproduction about every 60 days. Or it may be associated with egg and pupa cannibalism. Probably both factors are involved. The basic pattern is present in all the individual curves of Figure 4, although there is considerable difference as to the time of a high or a low count.

TABLE 4

COMPOSITION AND TOTAL SIZE OF *Gnathoceros* CONTROLS IN PERCENTAGE

($n = 129$)

	Mean	S D	C V
Larvae	83 4± 0 51	8 6 ± 0 36	10 3
Pupae	2 5± 0 17	2 9 ± 0 12	118 7
Male imagoes	6 3± 0 21	3 58± 0 15	56 6
Female imagoes	7 8± 0 21	3 5 ± 0 15	45 1
Total population	762 5±16 2	273 3 ±11 5	35 8

From the point of view of the experiments to follow, however, *Gnathoceros* can be thought of as maintaining its populations at a mean of about 762 (± 16.23) beetles per 100 gm. of medium. The standard deviation shows that two-thirds of the time the population counts do not exceed 1,035 beetles or fall below 489 beetles. The percentages of Table 4 show that the larvae constitute the bulk of the population (83 per cent) and vary little from that figure. The imagoes contribute, on the average, about 14 per cent to the total population, but they are much more variable in their distribution than the larvae. The pupae are sporadic in appearance. They may be present in some counts and completely absent in others. This is due to the short duration of the stage and to the fact that the pupae are heavily cannibalized.

Table 5 suggests that the sex ratio of *Gnathoceros* imagoes deviates from an expected 1:1 frequency. Since practically nothing is known about this point, the sex ratios in each of the eight populations were examined individually. These data appear in Table 5. There it is shown that in six of the eight cultures there are significantly more females than males. In one of the cultures the difference in favor of females is borderline ($P = 0.0918$), and in one the ratios do not deviate significantly from an expected frequency.

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[3] At the time of analysis two of the cultures were 720 days old; two, 690 days, two, 660 days; one, 600 days; and one, 390 days.

TABLE 5

SEX RATIO ANALYSIS OF *Gnathoceros* POPULATIONS

Mating	Male Imagoes (Percentage)	Female Imagoes (Percentage)	Imagoes Examined	Probability that the Sex Ratios Differ from a 1:1 Expectation
B¹	45.3	54.7	2,146	<0.0000
B²	50.3	49.7	2,670	>0.5000
B³	44.0	56.0	1,244	<0.0000
B⁴	43.9	56.1	2,334	<0.0000
B⁵	46.9	53.1	731	0.0918
B⁶	39.7	60.3	1,496	<0.0000
B⁷	42.9	57.1	1,289	<0.0000
B⁸	40.8	59.2	882	<0.0000
Mean	44.8	55.2	12,792	<0.0000

TABLE 6

Gnathoceros CONTROLS ARRANGED IN BODENHEIMER'S CLASSIFICATION

(A) Age	(B) Mean Total Population	(C) Mean Imagoes		(D) Potential Increase of the Population per Past 30 Days	(E) Rate of Growth	(F) Intensity of the Struggle for Existence
		Males	Females			
30	1,855.0	42.5	38.5	2,310.0	. .	. . . . . .
60	812.0	44.3	36.3	2,178.0	0.44	4,950.0
90	1,148.0	56.0	51.3	3,078.0	1.41	2,183.0
120	666.7	47.7	46.3	2,778.0	0.58	4,789.0
150	936.5	61.2	62.3	3,738.0	1.40	2,670.0
180	592.5	46.2	58.3	3,498.0	0.63	5,552.4
210	825.7	56.0	64.5	3,870.0	1.39	2,784.2
240	713.8	65.5	64.7	3,882.0	0.86	4,514.0
270	622.8	57.0	60.6	3,636.0	0.87	4,179.3
300	732.4	53.7	66.8	4,008.0	1.17	3,425.6
330	488.3	54.7	58.6	3,516.0	0.67	5,247.8
360	891.7	49.4	58.1	3,486.0	1.83	1,904.9
390	678.9	38.6	55.8	3,348.0	0.76	4,405.3
420	915.4	37.7	50.7	3,042.0	1.35	2,253.3
450	726.3	39.7	52.3	3,138.0	0.79	3,972.2
480	806.7	40.4	52.8	3,168.0	1.11	2,854.1
510	720.3	42.0	53.8	3,228.0	0.89	3,627.0
540	840.4	40.9	52.7	3,162.0	1.17	2,702.6
570	657.6	34.1	49.7	2,982.0	0.78	3,823.1
600	655.1	33.4	51.7	3,102.0	0.99	3,133.3
630	762.7	37.2	56.2	3,372.0	1.16	2,906.9
660	658.5	38.7	51.7	3,102.0	0.86	3,607.0
690	745.3	37.3	47.7	2,862.0	1.13	2,532.7
720	700.5	31.0	55.0	3,300.0	0.94	3,510.6

The mean of all eight populations shows that the females are decidedly more numerous than the males. Thus it appears that a real sex differential exists for *Gnathoceros* in most of these cultures. The difference cannot be attributed to faulty recognition, since the males are distinguished readily from females on the basis of their great mandibular development. Whether the difference represents a sex-linked lethal effect or some environmental selection remains to be explored.

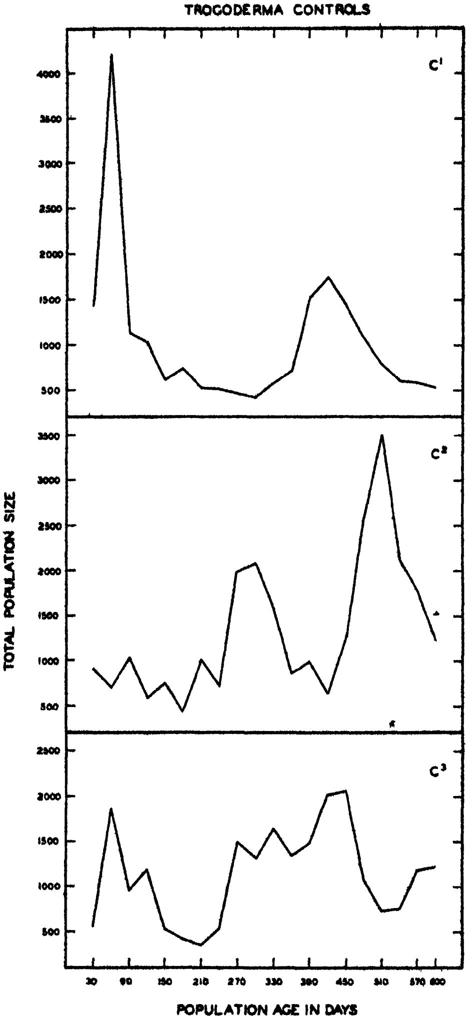

FIG. 5.—Population growth of *Trogoderma* controls. Curves based on individual cultures.

In Table 6 the *Gnathoceros* data are tabulated according to Bodenheimer's method. On the basis of original fecundity assays (unpublished) it was determined that *Gnathoceros* females produce on the average about 60 eggs per 30-day period. This figure is used in calculating Column D. Since the number of females is known, the potential increase per 30-day period is sixty times the number of females. From Table 6 the following facts are apparent: (1) *Gnathoceros* is much less productive than *Tribolium*. This shows in the total population size, the number of imagoes, the potential increase, and the intensity of the struggle for existence. (2) So far as the struggle for existence is a criterion, the total competitive pressure within a *Gnathoceros* population is less than it is for *Tribolium*. Obviously, this does not mean that an individual *Tribolium* contributes more to this pressure than an individual *Gnathoceros*. It means only that the *Tribolium* build up heavier cultures. (3) The rate of growth is a good index of the "high-low" characteristic mentioned earlier. In Column E there are twenty instances where a high rate of growth is followed by a low rate (or conversely) and only two instances where such is not the case.

Trogoderma versicolor.—The data on *Trogoderma* population growth are based on census observations made every 30 days over a 600-day span on three different cultures. The growth trend computed as a mean of these records is shown in Figure 1, and the individual curves appear in Figure 5. It is apparent that the curve for the grouped *Trogoderma* cultures has a different pattern from that of *Gnathoceros* and a somewhat less different pattern than *Tribolium*. As we have seen, the *Tribolium* curve oscillates over long periods, and the *Gnathoceros* curve maintains itself close to an average value. *Trogoderma* is much more variable than either of the other two species. In C^1 and C^3 it starts out low, rises quickly to a maximum, and then fluctuates erratically around a mean. The C^2 culture does not exhibit an initial spurt but builds up later. Clearly, some of these fluctuations are due to the small size of the sample, and some may be due to the technical error of losing certain eggs in the sifting process. It seems likely, however, that the major factor concerns the beetles' reproduction. Dick (1937) classified the oviposition of Coleoptera into four groups: Types "I, II, III and IV." Types I and II fit the species used in these investigations and are defined by Dick (p. 765) as follows:

> In Type I ". . . . belong species which live only a short time as adults and lay all their eggs within a few days." Examples, *Bruchus obtectus*, *Lasioderma serricorne*, *Trogoderma versicolor*.
>
> In Type II ". . . . is represented by species which live for a long time—often over a year—as adults, and produce eggs continuously over a long time." Examples, *Gnathoceros cornutus*, *Tribolium* sp., *Necrobia rufipes*.

It seems apparent that *Trogoderma* populations have a different growth form than *Tribolium* or *Gnathoceros*, largely owing to the oviposition pattern and short imaginal life. In Figure 1 at the 30-day count the total population is a function of the original 88 imagoes only. The latter are all dead at 30 days. Norris (1936) has shown that the mean duration of life of males is about 19 days and of females about 11 days. Our own observations based on smaller numbers are somewhat different.[4] Between the 30- and 60-day reading a second brood of imagoes typically emerges and then reproduces. This accounts for the rise at the time of the second census noticeable in C^1 and C^2. At 90 days the imago number is down again, and the population is declining. This decline continues with variations until, between the 240- and 270-day census, another sizable stock of imagoes gets established and greater reproduction ensues. From then on, alternation of imagoes with larvae and eggs gets "out of step," and the cultures maintain themselves at a higher level. Even so, there remains a sporadic character to the growth that is not found in *Tribolium* or *Gnathoceros*. We cannot suggest that all *Trogoderma* populations follow the precise form shown in Figure 1. The curves of Figure 5 show that this would be an erroneous assumption. Differences in handling, in stocks, environmental control, and coincidence all affect the curve. However, it is important to correlate the explosive nature of the growth with the reproductive characteristics of the imagoes. This is sound procedure in any population analysis.

The data on total population size and composition are presented in Table 7. The mean *Trogoderma* population over 600 days is 1,362 beetles per 100 gm. of medium. In two-thirds of the instances this figure does not go above 2,309 or below 415 beetles. As would be anticipated from the above discussion, this is a greater relative variation in population size than that exhibited by the two Tenebrionid species. This point is supported by the

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[4] We have obtained the following mean life-duration figures for *Trogoderma versicolor* imagoes kept in whole-wheat flour (sifted through an 82-mesh sieve) plus 2 per cent yeast:

SERIES	LIFE-DURATION (DAYS)		*n*
	Males	Females	
Grouped (14 pairs in 20 gm. medium)	7 7	11 1	14
Isolated (1 imago per 2 gm. medium)	11 0	16 2	39 (males) 37 (females)

coefficients of variability, which are 39 per cent for *Tribolium*, 35 per cent for *Gnathoceros*, and 69 per cent for *Trogoderma*. Table 7 shows that the larvae constitute about 90 per cent of the population, with the imagoes next and the pupae last.

In Table 7 there is a suggestion that *Trogoderma* males appeared more frequently in the counts than did *Trogoderma* females. This is tested in Table 8, where it turns out that in each of the three populations the difference is greater than could be accounted for on chance alone. Here, then, is a sex-ratio distortion in the opposite direction from that presented for *Gnathoceros*. Again, the causal factors are not known. Since male *Trogoderma* can be distinguished from females only because they are smaller, it was decided to test the validity of this criterion. One hundred *Trogoderma* imagoes were removed at random from a stock culture. These beetles were sexed independently by the three authors. Then the beetles were killed, their sex determined by dissection (47 males; 53 females), and compared with the results made on the basis of body size. The agreement between all three authors and the actual sex ratio was perfect. From these results it is apparent that sex was determined with complete accuracy by size differentials. Thus, although there may be an occasional negligible error, the method of sexing used in these experiments is reliable.

TABLE 7

COMPOSITION AND TOTAL SIZE OF *Trogoderma* CONTROLS IN PERCENTAGE

($n=68$)

	Mean	S.D.	C.V.
Larvae..........	89.4 ± 0.98	12.0 ± 0.69	13.4
Pupae...........	1.8 ± 0.18	2.2 ± 0.13	122.9
Male imagoes....	5.5 ± 0.33	4.1 ± 0.23	73.7
Female imagoes..	3.3 ± 0.20	2.5 ± 0.14	76.2
Total population.....	1361.9 ± 77.4	946.7 ± 54.0	69.5

TABLE 8

SEX RATIO ANALYSIS OF *Trogoderma* POPULATIONS

Population	Male Imagoes (Percentage)	Female Imagoes (Percentage)	Imagoes Examined	Probability that the Sex Ratios Differ from a 1:1 Expectation
C^1............	58.9	41.1	3,221	0.0000
C^2............	64.1	35.9	4,259	0.0000
C^3............	60.3	39.7	1,764	0.0000
Total......	61.4	38.6	10,407	0.0000

Table 9 arranges the *Trogoderma* data in the form suggested by Bodenheimer. There was some difficulty in arriving at the best way to express potential increase, since the imagoes live less than 30 days. Finally it was decided to take the mean number of females per 30-day interval and multiply this figure by 122 eggs. The latter is based on Norris' (1936) report that *Trogoderma* produced 122 eggs per 11 days, which is the mean length of life of females. To illustrate by an example, suppose we wish to compute the potential increase that exists between the 240- and the 270-day reading. The mean number of females that lived over this period is 23.85 (i.e., 7.0 + 40.7 ÷ 2). Thus 23.85 × 122 = 2,910 eggs. The potential-increase and struggle-for-existence columns of Table 9 bring out the erratic character of *Trogoderma*'s population dynamics. Both of these indices build up rapidly, sink to an early low point when the imagoes decline, and then build up as the population matures.

TABLE 9

Trogoderma CONTROLS ARRANGED IN BODENHEIMER'S CLASSIFICATION

(A) Age	(B) Mean Total Population	(C) Mean Imagoes		(D) Potential Increase of the Population per Past 30 Days	(E) Rate of Growth	(F) Intensity of the Struggle for Existence
		Males	Females			
30	967.3	0.0	0.0	....	....	....
60	2,387.7	151.3	100.3	6,118	2.47	2,476.9
90	1,114.0	60.7	25.7	7,686	0.47	16,353.2
120	956.7	19.3	13.0	2,361	0.86	2,745.3
150	628.0	5.0	5.3	1,116	0.66	1,690.9
180	546.7	9.0	4.0	567	0.87	651.7
210	650.3	11.7	7.3	689	1.19	579.0
240	594.3	32.0	7.0	872	0.91	958.2
270	1,378.0	61.7	40.7	2,910	2.32	1,254.3
300	1,356.7	113.0	41.0	4,984	0.98	5,085.7
330	1,396.7	94.0	68.7	6,692	1.03	6,497.1
360	1,020.7	63.0	26.4	5,801	0.73	7,946.6
390	1,491.3	136.7	105.7	8,058	1.46	5,519.2
420	1,571.3	102.7	65.0	10,413	1.05	9,917.1
450	1,702.0	132.0	69.0	8,174	1.08	7,568.5
480	1,758.7	134.3	89.0	9,638	1.03	9,357.3
510	1,983.3	203.3	152.0	1,470	1.13	1,300.9
540	1,238.3	120.0	64.7	13,219	0.62	21,321.0
570	1,363.7	138.3	72.0	8,339	1.10	7,580.9
600	1,088.0	99.7	62.3	8,192	0.80	10,240.0

Comparison of Tribolium, Gnathoceros, and Trogoderma controls.—Before proceeding to the interspecific experiments, the data for the three species growing as single-species cultures can be summarized as follows:

1. Growth trends (Figs. 1, 3, 4, and 5).
   a) *Tribolium* exhibits a relatively smooth cyclic oscillation during its first 420 days of husbandry. Thereafter, there is some evidence of a dampened oscillation.
   b) *Gnathoceros*, after an initial spurt, settles down to an average value and maintains itself closely about that average.
   c) *Trogoderma* grows erratically. There is a high spurt followed by a low region and then a higher period of great variability.
   d) All three species grow successfully (i.e., do not die out) under the conditions of husbandry described.

2. Total size (Tables 1, 4, and 7). On the average, *Tribolium* maintains the largest cultures (2,538.5 ± 61.4 beetles per gm. medium); *Trogoderma* comes next (1,361.0 ± 77.4 beetles); and *Gnathoceros* populations are smallest (762.5 ± 16.23 beetles). As regards variability of census counts, *Trogoderma* is the most variable, *Tribolium* next, and *Gnathoceros* least.
3. Composition (Tables 1, 4, and 7; Fig. 6).
   a) In all three species the larvae constitute the bulk of the population (65 per cent for *Tribolium;* 83 per cent for *Gnathoceros;* and 89 per cent for *Trogoderma*).
   b) The relative frequency of imagoes is highest for *Tribolium* (27.3 per cent), next for *Gnathoceros* (14 per cent), and lowest for *Trogoderma* (8.8 per cent).
   c) The pupae are always relatively sparse and may be completely absent from any one count. The mean per cents are for *Tribolium* 7.2, for *Gnathoceros* 2.5, and for *Trogoderma* 1.8.
   d) The variability is indexed by the standard deviations and coefficients of variability tabulated in Tables 1, 4, and 7. These parameters should be studied.

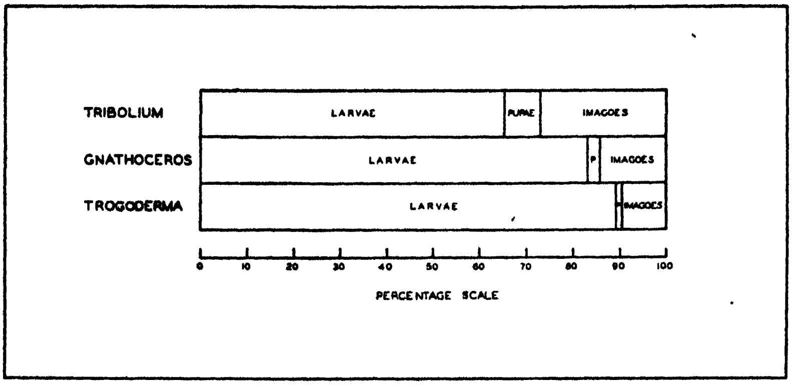

FIG. 6.—Percentage composition of control populations of *Tribolium, Gnathoceros,* and *Trogoderma*

   e) As to imaginal sex ratio, *Tribolium* does not deviate significantly from a 1 : 1 expectation; *Gnathoceros* produces on the average 44.8 males to 55.2 females, and *Trogoderma* produces 61.4 males to 38.6 females. The latter two deviations are significant statistically.

THE INTERSPECIFIC POPULATIONS

Tribolium and Gnathoceros.—The data on which the *Tribolium-Gnathoceros* interactions are based consist of (1) three matings (D^1, D^2, D^3) in which both species were started in equal density; (2) one mating (G) in which the initial density was 76 *Tribolium* to 12 *Gnathoceros*, and (3) one mating (I) in which the initial density was 12 *Tribolium* to 76 *Gnathoceros*. Since the *Tribolium-Gnathoceros* data are the most complicated, as well as the most interesting in many ways, we shall discuss them in some detail. The data are summarized in Tables 10 (D^1), 11 (mean of D^2 and D^3), and 12 (G, I) and graphed in Figures 7, 8, and 9. As an index of growth changes from one census to the next a simple ratio column appears in all the tables. In the present matings this index is computed always by dividing the total number of *Tribolium* for any particular census by the total number of *Gnathoceros*. Thus the index starts with a quotient value of 1 (44/44) in D; a value of 6.34 (76/12) in G; and a value of 0.16 (12/76) in I. The quotient is easily interpreted. As it increases in magnitude, *Tribolium* is besting *Gnathoceros;* as it decreases, *Gnathoceros* is driving out *Tribolium.*

First let us consider the D-matings. Figure 7 shows that two types of end results take place despite the fact that the experiments are all started in the same way. In graph D^1 *Tribolium* and *Gnathoceros* are about even in numbers at 30 days. Thereafter, *Tribolium* rises steadily and reaches, between 120 and 210 days, its first high point.

TABLE 10

Gnathoceros-Tribolium COMPETITION IN THE D^1-MATING

Age	*Tribolium*	*Gnathoceros*	Ratio $\frac{T}{G}$
1.........	44	44	1 00
30........	1,319	1,176	1 12
60...	974	404	2 34
90..	2,299	158	14 55
120....	3,002	51	58 86
150 . .	2,775	25	111 00
180....	2,947	5	589 00
210..	2,970	1	2,970.00
240..	2,465	0	....
270 .	2,030	....	....
300...	1,641	....	....
330..	4,133	....	....

TABLE 11

Gnathoceros-Tribolium COMPETITION: MEAN OF THE D^2-D^3–MATINGS (COMPUTED FOR ALL STAGES)

AGE	LARVAE		RATIO $\frac{T}{G}$	PUPAE		RATIO $\frac{T}{G}$	IMAGOES		RATIO $\frac{T}{G}$	TOTAL		RATIO $\frac{T}{G}$
	Trib.	*Gnath.*		*Trib*	*Gnath.*		*Trib.*	*Gnath*		*Trib.*	*Gnath.*	
1...	....	.....	...	.....	.....	......	44.0	44 0	1 00	44 0	44.0	1.00
30...	1,226.0	1,046.5	1 17	413 5	0 0	......	62 5	37 5	1 67	1,702 0	1084 0	1.57
60...	1,550 5	190.0	8 16	81.5	28 0	2.91	325 5	36 5	8 92	1,957 5	254.5	7 69
90...	1,828 5	196.5	9 30	152 0	5 5	27 63	428 5	42 0	10 20	2,409 0	244 0	9 87
120....	1,529.5	163 0	9.38	60.5	10 0	6 05	380 5	29 0	13.12	1,970.5	202 0	9 75
150....	1,394.5	116.5	11 97	52 5	1.0	52 50	320 0	20 5	15 61	1,767.0	138.0	12 80
180....	1,727.0	86 5	19 97	44 0	1.5	29 33	249.5	25 0	9 98	2,020.5	113.0	17 88
210....	597.0	176 0	3.39	31 0	8 5	3 64	203.0	39 0	5 20	831 0	223.5	3 72
240....	339 5	348.5	0.97	26.5	13.5	1 96	145.0	36 0	4.03	511.0	398.0	1.28
270....	104 0	398.5	0.27	2 0	11.5	0 17	87 5	44 0	1 99	193.5	444.0	0 43
300....	19 0	613.5	0.03	14.5	4.0	3 62	60.5	56 5	1 07	94 0	674.0	0.14
330....	1.0	394 0	0.003	1.0	17 0	0.06	34 0	58 5	0 58	36.0	469.5	0.08
360....	2.0	662.5	0.003	0 0	13.5	....	29.0	66 0	0.44	31.0	742.0	0.04
390....	0.5	502.0	0 001	0 0	18.5	......	22.5	57.5	0 39	23.0	578.0	0.03
420....	0.0	870.5	......	0 0	12.5	......	15.0	64 5	0 23	15.0	947.5	0 01
450....	0.0	649.5	......	0.0	8 0	....	12.0	58 5	0.21	12.0	716.0	0 01
480....	0.0	883.0	......	0.0	4 0	......	6 0	91 5	0 07	6.0	978.5	0 006
510....	0.0	554.0	......	0 0	4 0	.....	3 5	81 5	0.04	3.5	639 5	0.005
540....	0.0	801.5	....	0.0	11.5	.....	3 0	102 5	0 03	3 0	915 5	0.003
570....	0.0	550.5	......	0.0	10 5	....	1 5	97.0	0.01	1 5	658.0	0 002
600....	0.0	684.5	....	0.0	7.0	....	0 0	111.5	....	0.0	803 0	......

During this time *Gnathoceros* is consistently going down. By 240 days all *Gnathoceros* are gone, and the culture is now a pure *Tribolium* population. Since *Gnathoceros* controls maintain themselves indefinitely and since nothing has been altered except the introduction of *Tribolium*, we must conclude that in the D¹-mating the latter species drives out *Gnathoceros* with efficiency and dispatch. A glance at Figure 7 and Table 10 shows that *Tribolium* goes on in typical control fashion with a drop at 300 days and a sharp rise at 330 days. In short, the 210-day association with *Gnathoceros* does not affect the subsequent course of the population.

Figure 7, graphing the mean[5] of D² and D³, and Figure 8, depicting these cultures individually, present a different picture in the sense of the end result. Yet the interpretation seems consistent with that discussed above. In both D² and D³, *Tribolium* rapidly gets ahead of *Gnathoceros* and reaches a peak that extends between 90 and 180 days. During this time *Gnathoceros* is steadily declining. This decline is a repetition of the events of D¹. The reason that D² and D³ differ from D¹ seems to be due to the following two factors, both of which pertain to *Tribolium* growth only: (1) The *Tribolium* populations of D² and D³ do not reach so great a density over the 90–180-day intervals as did D¹ (means = 2,041 beetles for D², D³; 2,756 beetles for D¹), and (2) the decline in *Tribolium* comes earlier in the D², D³ than in the D¹ culture. In Table 11 note that, as *Tribolium* starts to decline owing to some factor not connected with its competitor, *Gnathoceros* immediately starts to build up. The total-population column at 180 days shows *Tribolium* has a count of 2,020 and *Gnathoceros* 113. The latter figure is the lowest

TABLE 12

Gnathoceros-Tribolium COMPETITION FOR THE G¹- AND I¹-MATINGS

AGE	G¹-MATING			I¹-MATING		
	Tribolium	*Gnathoceros*	Ratio $\frac{T}{G}$	*Tribolium*	*Gnathoceros*	Ratio $\frac{T}{G}$
1	76	12	6.34	12	76	0.16
30	2,695	366	7.36	316	1,406	0.22
60	2,264	162	13.97	82	732	0.11
90	3,374	21	160.00	935	664	1.41
120	3,010	7	430.00	434	386	1.12
150	2,745	10	274.00	1,738	392	4.43
180	2,925	6	487.00	2,027	301	6.73
210	2,596	3	865.00	1,260	165	7.64
240	1,812	1	1,812.00	2,436	110	2.21
270	1,149	1	...	1,224	185	6.67
300	1,021	1	...	521	250	2.08
330	1,243	0	...	277	603	0.46
360	2,520	...	...	102	925	0.11
390	2,890	...	...	49	771	0.06
420	3,456	...	...	49	783	0.06
450	...	...	...	17	563	0.03
480	...	...	...	13	527	0.02
510	...	...	...	10	951	0.01
540	...	...	...	6	754	0.008
570	...	...	...	6	651	0.009
600	...	...	...	4	693	0.006
630	...	...	...	3	677	0.004
660	...	...	...	3	507	0.005

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[5] In certain cases replicated experiments have been grouped and averaged. This has been done only in those instances where the experiments are basically similar in trend and end result.

that *Gnathoceros* reaches. Between 180 and 210 days *Tribolium* drops to 831 beetles. During the same interval *Gnathoceros* increases from 113 to 223! In a control culture *Tribolium* would build up again. Instead it goes down steadily and finally becomes extinct at 600 days. *Gnathoceros* rapidly attains control size and thereafter displays the short high-low trend characteristic of its growth. In sum, it appears that there is no real distinction in terms of causes between D^1 and D^2–D^3. In both cases the end results depend on what happens to *Tribolium*. *Tribolium* is not affected by *Gnathoceros* until it reaches a density that is abnormally low. If it gets to this low density, then *Gnathoceros* becomes a successful competitor, and the flour beetle is slowly driven out. These D-experiments suggest that the initial density is not necessarily the important factor in controlling the future of the population. It is the numerical "establishment" of *Tribolium* that is significant.

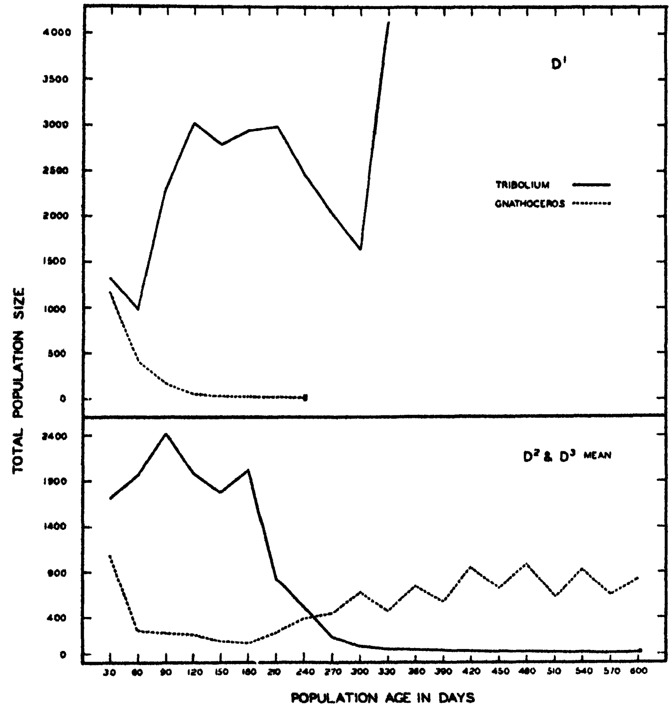

FIG. 7.—Population growth in the *Tribolium-Gnathoceros* cultures. Upper graph, D^1; lower graph, mean of D^2 and D^3.

The points just made for the D-matings are supported by the G- and I-matings graphed in Figure 9 and summarized in Table 12. Let us consider first the G case where the imagoes were introduced in the ratio of 76 *Tribolium* to 12 *Gnathoceros*. Here the data are clear cut. The *Tribolium*, as a consequence of their superior initial density, immediately establish themselves and build up a large population by the first census (2,695 beetles). The mean number of *Tribolium* over the 90–180-day interval is 3,013. The *Gnathoceros*, having made a feeble start at best (366 beetles at 30 days), are competing with a vigorous *Tribolium* population. The result is that they immediately and rapidly start to decline and are extinct by the time *Tribolium* reaches its low point. In this case there is never an opportunity for successful competition on the part of *Gnathoceros*; they are bested from the start.

In the I-mating *Gnathoceros* was started high and *Tribolium* low. The drop in *Gnathoceros* during the 30–60-day interval is characteristic of the controls and cannot be considered as primarily due to *Tribolium*. However, after 60 days *Tribolium* begins to build up. The imagoes increase from the start of the experiment up to the 210-day count. This is important because they are the reproducing element in the population and they do not seem to be restrained by their competitor. Apparently, *Gnathoceros* is not a successful competitor of *Tribolium* during the early stage of the culture, when, presumably, the latter is not hindered by infection. As the *Tribolium* density increases, *Gnathoceros* goes down. This is the experience of the other matings. However, *Tribolium* has not established itself well enough during its first 240 days of life completely to drive out *Gnathoceros*, although the latter has fallen from 732 at the 60-day census to 110 at the 240-day census. At 240 days *Tribolium* starts its decline. The total density drops from 2,436 beetles to 1,224 at 270 days, to 521 at 300 days, to 49 (46 imagoes) at 390 days. *Gnathoceros* starts to rise between 240 and 270 days and attains its control density around 330 days. Thereafter, *Tribolium* goes slowly down until at 660 days only 3 imagoes remain. Between the 660- and 690-day reading something took place in the culture that complicates the picture. At the 690 count there were 26 *Tribolium* larvae, 7 pupae, and 7 imagoes. In other words, the *Tribolium* population had increased even in the face of *Gnatho-*

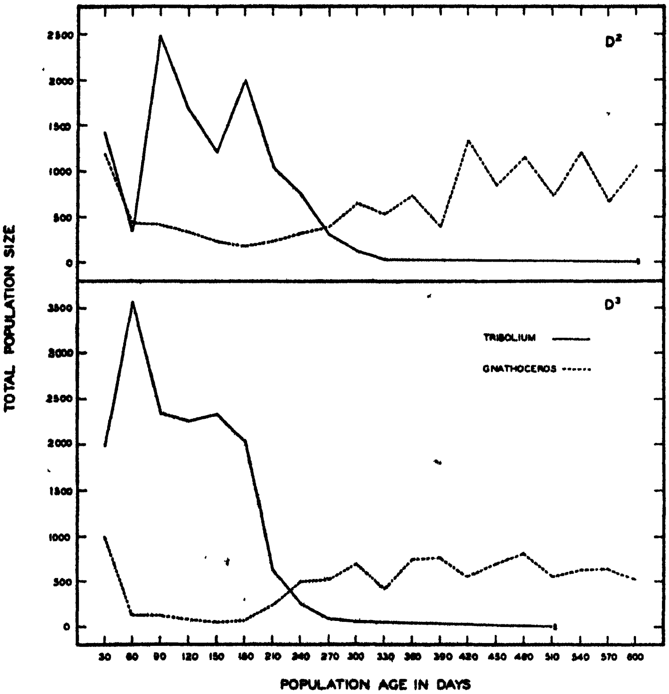

FIG. 8.—Population growth in the *Tribolium-Gnathoceros* cultures. Upper graph, D²; lower graph, D³

ceros pressure. Whether this increase represents a contamination (i.e., inadvertent entry of *Tribolium* from another source) or a real increase cannot be said. At the time of this writing the I-mating has been observed through the 750-day count, and the *Tribolium* have increased slightly. The culture will be continued until a definite conclusion can be drawn. That conclusion will be reported in the body of other experimental analyses.

It is interesting to note for matings D^2, D^3, and I, where *Tribolium* is bested by *Gnathoceros*, that it is the larvae and pupae that are driven out in greatest proportion and not the imagoes. The latter, although highly vulnerable to the supposed parasitic infection, apparently are relatively immune to the pressure of *Gnathoceros* competition. They constitute the small number that lingers on. Finally they die, presumably of old age. This suggests that *Gnathoceros* may function as a predator on *Tribolium* eggs, small larvae, and pupae. This predation becomes significant only when *Tribolium* imagoes die off and thus reduce the reproductive capacity of the population. The nature of the competition pressure exerted by *Tribolium* on *Gnathoceros* is not clear, but in part it may be a predation effect also.

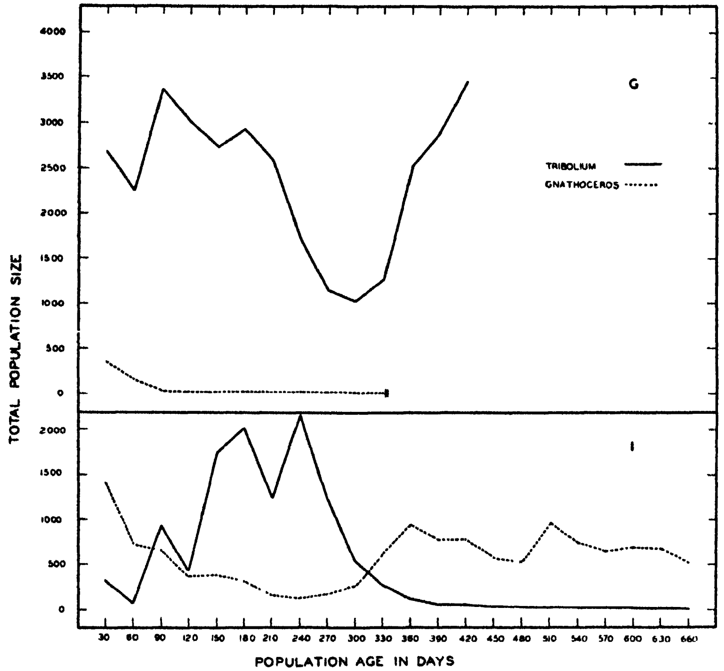

FIG. 9.—Population growth in the *Tribolium-Gnathoceros* cultures. Upper graph, G; lower graph, I

Recapitulation.—The most important points emerging from this experimental study of *Gnathoceros* and *Tribolium* in population association may be stated as follows:

1. If *Tribolium* gets well established as a total population, it is not affected dangerously by *Gnathoceros*. By "dangerously" we mean extinction or near-extinction, and we stress that point since there is some suggestion that *Tribolium* populations do not get so large when in association with *Gnathoceros* as they do otherwise.

]

2. A well-established *Tribolium* culture always drives out *Gnathoceros*.
3. If *Tribolium* is reduced to a certain point, it is driven out by *Gnathoceros*. The latter presumably have their competitive effect primarily on the eggs, larvae, and pupae.

TABLE 13

Tribolium-Trogoderma COMPETITION: MEAN OF THE E^1-E^2-MATINGS (COMPUTED FOR ALL STAGES)

AGE	LARVAE		RATIO Trib./Trog.	PUPAE		RATIO Trib./Trog.	IMAGOES		RATIO Trib./Trog.	TOTAL		RATIO Trib./Trog.
	Trib.	*Trog.*		*Trib.*	*Trog.*		*Trib.*	*Trog.*		*Trib.*	*Trog.*	
0–1....	......	......	......	......	......	......	44	44	1.00	44	44	1.00
30....	1,765	910	1.94	302	0	......	40	0	......	2,107	910	2.31
60....	1,660	1,031	1.61	94	16	5.87	848	197	4.30	2,602	1,244	2.09
90....	2,432	89	27.32	130	......	......	882	182	4.85	3,444	271	12.71
120....	2,145	10	214 50	92	......	......	734	......	......	2,971	10	297.10
150....	2,644	0	......	65	......	......	674	......	......	3,383	......	......
180....	2,180	......	......	26	......	......	603	......	......	2,809	......	......
210....	1,935	......	......	144	......	......	512	......	......	2,591	......	......
240....	1,836	......	......	147	......	......	380	......	......	2,363	......	......
270....	988	......	......	205	......	......	349	......	......	1,542	......	......
300....	1,560	......	......	342	......	......	446	......	......	2,348	......	......
330....	2,257	......	......	265	......	......	462	......	......	2,984	......	......
360....	2,673	......	......	337	......	......	738	......	......	3,748	......	......

TABLE 14

Tribolium-Trogoderma COMPETITION IN THE H^1-H^2-MATINGS (MEAN) AND THE K^1-MATINGS

Age	*Tribolium*	*Trogoderma*	Ratio Trib./Trog.	*Tribolium*	*Trogoderma*	Ratio Trib./Trog.
1...............	76	12	6.34	12	76	0 16
30...............	3,347	120	27.9	1,157	983	1.18
60...............	4,368	47	92.9	2,395	683	3.51
90...............	3,783	1	3783.0	2,723	51	53.4
120...............	3,510	1	3510.0	3,230	16	201.9
150...............	3,254	0	....	2,512	9	279.1
180...............	2,784	....	....	2,448	4	612.0
210...............	2,359	....	....	1,990	2	995.0
240...............	1,817	....	....	1,465	2	732.5
270...............	939	....	....	1,314	1	1,314.0
300...............	1,060	....	....	2,216	0	....
330...............	2,005	....	....	2,734	....	....
360...............	3,173	....	....	2,561	....	....

In the experiments reported here the major reduction of *Tribolium* populations is not due to *Gnathoceros* but to those same factors that cause the oscillations observed in control cultures.

If *Tribolium* populations maintained a high asymptotic density without large oscillations, it seems likely that *Gnathoceros* would always be driven out.

Tribolium and Trogoderma.—The data on which the *Tribolium-Trogoderma* interactions are based consist of (1) two matings (E^1 and E^2) in which both species were started in equal density; (2) two matings (H^1 and H^2) in which the initial density was 76 *Tribolium* to 12 *Trogoderma;* and (3) one mating (K) in which the initial density was 12 *Tribolium* to 76 *Trogoderma*. The data are summarized in Tables 13 (mean of E^1 and E^2) and 14 (mean of H^1 and H^2; K) and graphed in Figures 10 (E), 11 (H^1 and H^2; K) and 12 (H^1; H^2).

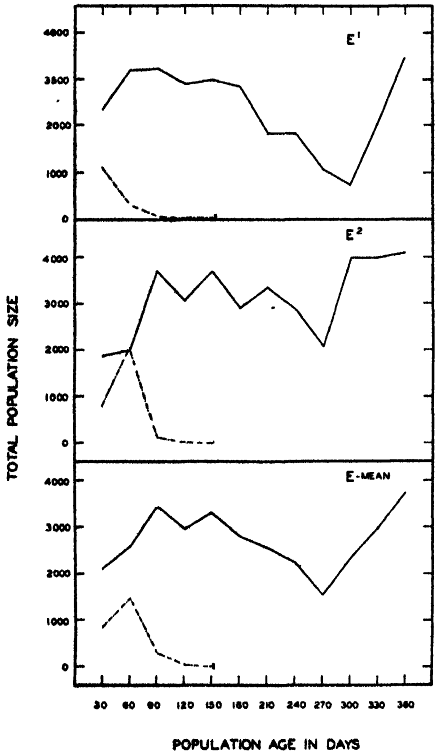

FIG. 10.—Population growth in the *Tribolium-Trogoderma* cultures. Upper graph, E^1; middle graph, E^2; lower graph, mean of E^1 and E^2. Solid line is *Tribolium*.

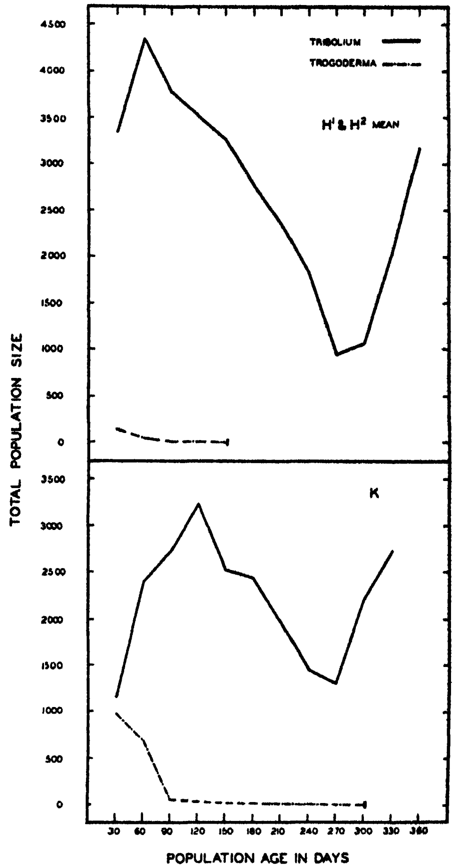

FIG. 11.—Population growth in the *Tribolium-Trogoderma* cultures. Upper graph, mean of H^1 and H^2; lower graph, K.

The major conclusion that emerges from these data is that *Tribolium* always drives out *Trogoderma*, irrespective of the initial-density conditions. Figure 10 and Table 13 show that, in the E-experiments, *Tribolium* starts out in normal fashion, reaches a mean population of 3,152 beetles between 90 and 180 days, and then declines. *Trogoderma* builds up during the first 60 days, and a new generation of imagoes emerges during the 30–60-

day interval. After 90 days all imagoes die out, and the last larvae disappear between the 120- and the 150-day interval. By 150 days the *Trogoderma* are extinct. Clearly, this extinction must be a function of some potent competitive pressure impinged on *Trogoderma* by *Tribolium*.

For the H-experiments, Table 14 and Figures 11 and 12 show that the *Tribolium* pressure is so rigorous that there is practically no effective reproduction on the part of the original imagoes and a second generation never emerges. Pragmatically speaking, the *Trogoderma* are extinct by 90 days, since only one larva in one culture lives during the 90–120-day interval. The H-experiments furnish an example of the drastic selection action of one species against another.

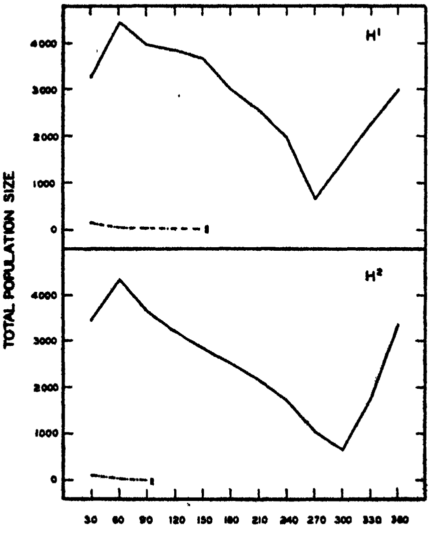

FIG. 12.—Population growth of *Tribolium-Trogoderma* cultures. Upper graph, H^1; lower graph, H^2. Solid line is *Tribolium*.

The K-experiment provides *Trogoderma* with a numerical advantage over *Tribolium* at the time the culture is started. In spite of this advantage *Trogoderma* is ineffectual as a competitor. The *Tribolium* population starts growing in characteristic fashion and reaches during the 90–180-day interval a mean size of 2,728 beetles. Thereafter, decline sets in. *Trogoderma* builds a total population of 983 beetles by 30 days, and during the 30–60-day interval the F_1 imagoes totaling 260 appear. In the absence of *Tribolium* pressure *Trogoderma* now should be in a favorable position for expansion. It has a balanced distribution of larvae, pupae, and imagoes. Instead, between 60 and 90 days, its population is reduced from a 683 to a 51 total. Only larvae remain; all pupae and imagoes are gone. The larvae are rapidly reduced between 90 and 120 days and then die off until they become extinct during the 270–300-day interval.

Summary.—The major points of the *Tribolium-Trogoderma* competition can be summarized as follows:

1. *Tribolium* always drives out *Trogoderma*.
2. The rate of extinction of *Trogoderma* is roughly proportional to the initial density of *Tribolium*.
3. *Trogoderma* produces an F_1 imago generation unless the early (0–60-day) pressure of *Tribolium* is exceedingly great. In the latter case (H), the only *Trogoderma* reproduction, is a product of the initial imagoes.
4. Once *Trogoderma* starts its decline, it goes out rapidly.
5. *Trogoderma* probably has some effect on the *Tribolium* populations. This point must await more specific analysis before definite statements are possible.

Gnathoceros and Trogoderma.—The data on which the *Gnathoceros-Trogoderma* interactions are based consist of (1) one mating (F) in which both species were started in equal density; (2) one mating (J) in which the initial density was 76 *Gnathoceros* to 12

Trogoderma; and (3) one mating (L) in which the initial density was 12 *Gnathoceros* to 76 *Trogoderma*. The data are summarized in Tables 15 (F) and 16 (J; L) and graphed in Figure 13.

In its most general form the *Gnathoceros-Trogoderma* competition can be summarized by pointing out that, of the two species, *Gnathoceros* is somewhat the stronger and usually, although not invariably, wins. The critical factor seems to be "establishment"—the degree to which one species gets an advantage over the other.

In mating F, where both species are started in equal density, *Gnathoceros* wins out after 750 days. It builds up its usual heavy larval population (1,174) during the first 30 days of culture. For the same interval the *Trogoderma* population is low (241 larvae), the 44 seeded imagoes have died, and the F_1 generation has not yet appeared. Between 30 and 60 days both species drop; some *Trogoderma* imagoes emerge and oviposit, and *Gnathoceros* is beginning to pupate. At 90 days new reproduction has bolstered both species, and both are in a favorable position for further growth. At 120 days the two have dropped but still are about evenly matched. During the 120–50-day interval, however, *Gnathoceros* attains the telling start over *Trogoderma*—not in the sense that it builds up a population of much greater density but in the sense that *Trogoderma*, by losing its imagoes, thereby loses its reproducing power. From then on *Trogoderma* gradually but surely dwindles. It is not a fecund culture. *Gnathoceros* assumes its control

TABLE 15

Gnathoceros-Trogoderma COMPETITION IN THE F-MATING (COMPUTED FOR ALL STAGES)

AGE	LARVAE		RATIO $\frac{G}{T}$	PUPAE		RATIO $\frac{G}{T}$	IMAGOES		RATIO $\frac{G}{T}$	TOTAL		RATIO $\frac{G}{T}$
	Gnath.	*Trog*		*Gnath*	*Trog.*		*Gnath.*	*Trog*		*Gnath.*	*Trog.*	
0–1	...	...	...	.	.	...	44	44	1 00	44	44	1.00
30	1,174	241	4 87	0	0	...	38	0	...	1,212	241	5.03
60	200	86	2 33	23	10	2 30	31	28	1 11	254	124	2.05
90	841	652	1 29	1	0		92	23	4 00	934	675	1 38
120	198	413	0.48	16	2	8 00	104	51	2 04	318	466	0 68
150	263	463	0.57	1	0		109	1	109 00	373	464	0 80
180	127	253	0 50	10	0	...	86	6	14 33	223	259	0.86
210	146	201	0 73	4	0	...	86	5	17 20	236	206	1 14
240	296	167	1.77	1	0	...	66	0	...	363	167	2.17
270	175	153	1 14	18	0	...	76	1	76 00	269	154	1.76
300	540	147	3 67	1	0	...	98	2	49 00	639	149	4.29
330	238	125	1 90	14	0	...	122	0	...	374	125	2 99
360	505	115	4 39	10	0	...	109	0	.	624	115	5.43
390	499	98	5 09	12	0	...	106	0	...	617	98	6.29
420	722	79	9 14	7	0	...	79	0	...	808	79	10 23
450	612	68	9 00	32	0	...	97	1	97 00	741	69	10.74
480	669	61	10.97	8	0	...	53	0	...	730	61	11 97
510	528	53	9.96	23	0	...	105	1	105 00	656	54	12.15
540	698	41	17 02	12	0	...	90	1	90 00	800	41	19.51
570	541	23	23 52	11	0	...	58	0		610	23	26 52
600	381	16	23 81	19	0	...	73	1	73 00	473	17	27 82
630	881	6	146.83	0	0	...	102	0	.	983	6	163.83
660	524	4	131.00	49	0	...	103	0	...	676	4	169.00
690	955	3	318 33	0	0	...	94	0	...	1,049	3	348 67
720	419	1	419 00	4	0	...	259	0	...	682	1	682 00

TABLE 16

Gnathoceros-Trogoderma COMPETITION IN THE J- AND L-MATINGS

Age	J-Mating			L-Mating		
	Gnathoceros	*Trogoderma*	Ratio $\frac{G}{T}$	*Gnathoceros*	*Trogoderma*	Ratio $\frac{G}{T}$
1	76	12	6.34	12	76	0.16
30	1,551	63	24.62	602	555	1.08
60	558	24	23.25	148	2,004	0.07
90	659	94	7.01	181	989	0.18
120	184	24	7 67	63	1,183	0 05
150	419	17	26 65	44	508	0 09
180	753	9	83.67	56	441	0.13
210	488	6	81 33	53	371	0 14
240	645	5	129 00	29	537	0.05
270	366	5	73.20	20	1,539	0 01
300	714	4	178 50	7	1,336	0 005
330	512	4	128.00	3	1,895	0 001
360	728	4	182 00	0	1,432	....
390	749	3	249 67	....	1,607	....
420	853	2	426.50	....	....	....
450	636	1	636 00	....	....	....
480	630	1	630 00	....	....	....
510	775	0	....	....	....	....

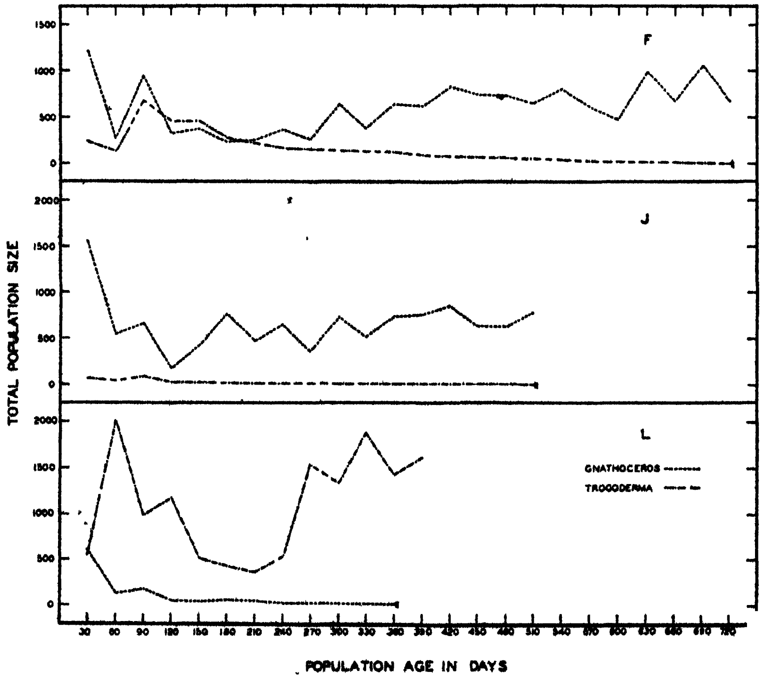

FIG. 13.—Population growth of *Gnathoceros-Trogoderma* cultures. Upper graph, F; middle graph, J; lower graph, L.

appearance by 300 days. For this case it thus appears that *Gnathoceros* competes successfully against *Trogoderma* by keeping the latter from building up an imaginal population.

In the J-mating *Gnathoceros* is started high and immediately gets well established. At 30 days there are 1,484 larvae and 67 of the original imagoes. Under this pressure *Trogoderma* fails to reproduce effectively. There is a feeble peak at 90 days due to the emergence of a few F_1 imagoes, but thereafter the population declines and becomes extinct by 480 days. In a sense *Trogoderma* is extinct at 90 days, for not one pupa or imago is produced during the 90–480-day interval. *Gnathoceros* assumes its control character by 150 days.

The L-experiment, although different in end result, correlates nicely with the above matings. By virtue of a high initial seeding, *Trogoderma* is successfully competing with *Gnathoceros* at 30 days and has built up a population of 555 larvae. During the 30–60-day interval 220 imagoes emerge. These reproduce with explosive force, and by 60 days a total population of 2,004 *Trogoderma* is counted. It is plain from the data that this high *Trogoderma* density is not without its effect on *Gnathoceros*, since the latter drop from 602 at 30 days to 148 at 90 days. *Trogoderma* is now well established, and its imagoes keep emerging and reproducing. In the face of this erratic pressure *Gnathoceros* dwindles, both as to larvae and as to adults, and by 360 days it is extinct.

Summary.—The major points of the *Gnathoceros-Trogoderma* competition can be summarized as follows:

1. *Gnathoceros* when started at equal or higher initial density drives out *Trogoderma*. The latter disappears faster in the J-mating than in the F.
2. *Trogoderma* seems to be driven out because it fails to keep enough imago generations emerging to reproduce itself. This suggests that *Gnathoceros* pressure in some way inhibits *Trogoderma*'s pupation and emergence.
3. *Trogoderma* can drive out *Gnathoceros*, if it gets well established. It seems particularly important that a large F_1 brood of imagoes emerges. The nature of the *Trogoderma* pressure on *Gnathoceros* is not clear.

The M-mating.—In addition to the two-species crosses, one mating was started with *Tribolium*, *Gnathoceros*, and *Trogoderma* introduced in the initial density of 30 each (15 males to 15 females). This mating was started primarily to test the technical feasibility of working with the three forms. However, since the results are of some interest in the light of the other experiments they are summarized briefly in Table 17 and Figure 14, where total population counts are listed for each species. The following points can be made:

1. *Trogoderma* becomes extinct in 120 days. In the face of such stringent competition there is essentially no reproduction detectable.
2. *Gnathoceros* and *Tribolium* develop somewhat erratically at first. The former has its usual large 30-day larval populations, and the latter is slow in getting started. During the 90–180-day interval *Tribolium* gets well established, and the *Gnathoceros* counts begin to decline. At the time of this writing the culture has run 450 days, and *Gnathoceros* is reduced to 8 larvae and 11 imagoes.

✓ *Competition and composition.*—In the earlier treatment of the single-species controls the composition of the populations was discussed. Also, in dealing with certain of the mixed populations, we pointed out here and there that one stage seemed to be affected by competition pressure while another stage seemed relatively immune. The latter were selected data; they covered events in the culture during a certain period of the life-cycle only, usually when the species was already well on its way to extinction.

TABLE 17

Tribolium-Gnathoceros-Trogoderma COMPETITION IN THE M-MATING

Age	*Tribolium*	*Gnathoceros*	*Trogoderma*
30	535	969	29
60	269	468	12
90	1,188	438	1
120	1,469	316	0
150	945	146	....
180	1,799	151	....
210	1,644	231	....
240	1,093	249	....
270	1,285	248	....
300	1,822	106	....
330	1,495	137	....
360	2,858	90	....
390	2,707	60	....
420	3,205	31	....
450	2,794	19	....

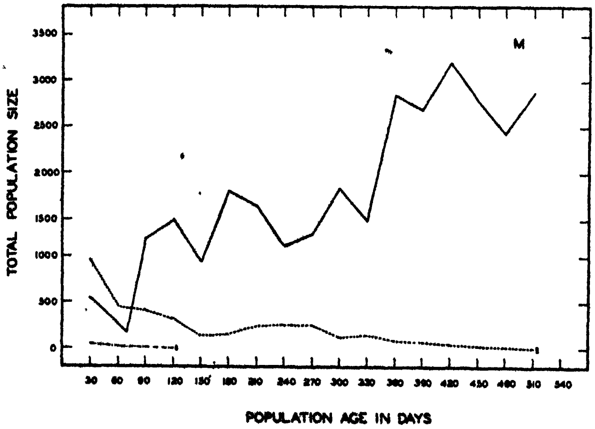

FIG. 14.—Population growth of *Tribolium-Gnathoceros-Trogoderma* culture (M-mating). Solid line, *Tribolium;* dotted line, *Gnathoceros.*

In this section we ask two questions: Is the composition during the total tenure of the experiment differentially altered depending on whether the species wins or loses to its associate, and are there any significant deviations in composition for any species when it is cultured with another form as compared with its control (isolated) growth? Both of these matters are considered in Table 18. This table tabulates experiment by experi-

TABLE 18

THE EFFECT OF COMPETITION ON COMPOSITION: SUMMARY OF ALL MATINGS FOR ALL THREE SPECIES

	Tribolium			*Gnathoceros*			*Trogoderma*		
	Larvae	Pupae	Imagoes	Larvae	Pupae	Imagoes	Larvae	Pupae	Imagoes
Percentage composition when species wins	68.1	5.1	26.2	88.2	1.8	10.0	91.5	1.2	7.3
	60.4	4.4	35.2	85.6	2.2	12.2			
	73.4	4.7	21.9	82.8	1.9	15.3			
	57.1	4.5	38.4	78.4	2.1	19.5			
	77.5	4.5	18.0						
Mean (winners)	67.4	4.7	27.9	83.7	2.0	14.3	91.5	1.2	7.3
Percentage composition when species loses	75.9	6.7	17.4	91.3	1.0	7.7	83.8	0.6	15.6
	60.4	4.4	35.2	90.3	0.2	9.5	99.4	0.0	0.6
				74.9	2.6	22.5	92.3	1.9	5.8
							96.3	0.3	3.4
							99.2	0.8	0.0
Mean (losers)	68.1	5.6	26.3	85.5	1.3	13.2	94.2	0.7	5.1
Mean (winners and losers)	67.6±2.01	4.9±0.18	27.5±2.04	84.5±1.45	1.7±0.18	13.8±1.30	93.7±1.71	0.8±0.2	5.5±1.43
Control (single species)	65.5±0.73	7.2±0.36	27.3±0.83	83.4±0.51	2.4±0.17	14.2±0.41	89.4±0.98	1.8±0.18	8.8±0.53

ment for each of the three species (1) the percentage of larvae, pupae, and imagoes when one species wins and the mean for that group; (2) the percentage when the same species loses and its mean; (3) the mean of winners *plus* losers; and (4) the control means taken from Tables 1, 4, and 7. It is recognized, of course, that the numbers are small—too small to add probable errors to certain means. This parameter is computed for the means of winners *plus* losers to allow comparison with control means. Despite the objection of small numbers the following points are worth mentioning:

1. There is no evidence in Table 18 that the percentage composition is affected by either winning or losing. There are some deviations, and, unfortunately, their significance cannot be appraised statistically. But at least we can say definitely that there are no marked alterations of composition that depend on the success or failure of a competition. In fact, the percentages are rather closer than one might expect a priori.
2. When the means for losers and winners are grouped (which procedure is permissible in the light of conclusion 1 just above) and compared with control means the differences in relation to their probable error result as shown in Table 19. These comparisons show that for *Tribolium* and *Gnathoceros* interspecific competition does not alter significantly the percentage of larvae or imagoes but seems to decrease the number of pupae, and for *Trogoderma* competition tends to increase the number of larvae and to decrease the number of pupae and imagoes. The latter findings check with earlier discussions of the *Trogoderma* matings.

TABLE 19

Stages	*Tribolium*	*Gnathoceros*	*Trogoderma*
Larvae	1.4	1.0	3.2
Pupae	8 5	4.4	5.5
Imagoes	0.1	0.4	3.2

DISCUSSION

Critique.—At this point it seems desirable to evaluate this investigation critically. Let us examine the technique employed and list its weaknesses and strengths as follows:

1. Debits
   - *a*) Although no eggs (except some of *Trogoderma*) are lost, egg counts cannot be made because of the nature of the medium. It would be desirable to know how egg numbers vary from one count to another. This might give a closer approximation of productivity, as well as some information on cannibalism.
   - *b*) More replications of certain matings would be desirable. Owing to the tedious character of the census method (one examination takes from 1 to 3 hours for two observers) it was impossible for us to maintain a more extensive experiment. Additional replications would smooth out the variation and strengthen the interpretations in certain spots. We do feel, however, that the investigation is extensive enough to warrant the drawing of accurate, descriptive conclusions.
   - *c*) There is a disturbance element introduced into the experiment when the census is taken. Patently, this cannot be avoided, but we have tried to standardize handling so that this disturbance is essentially minimal and equivalent for each mating. The general consist-

ency of the results suggests that we were fairly successful in this effort. Our experience indicates that *Trogoderma* is more vulnerable to handling effects than either *Gnathoceros* or *Tribolium*.

2. Credits
    a) In addition to the close standardization mentioned above, the environment external to the culture bottle is controlled. Also, there are no critical differences in environment between one mating and another, since all bottles are kept in the same incubator.
    b) By renewing the medium at each 30-day count, conditioning is kept from being an important variable in this study. The competition measured is thus an expression of beetle behavior in an essentially optimum environment.
    c) The medium itself is closely standardized and reproducible.
    d) There is an accurate collection, counting, and recording of the beetles. Although the technique is laborious, it does not sacrifice reliability.
    e) In taking the census the population is not sampled but is counted in entirety. This rules out a source of variation and permits working with larger numbers.

In summary it seems judicious to say that, while our technique does have some obvious faults and most certainly can be improved, it does, nevertheless, permit the collection of an accurate body of quantitative knowledge about a biological topic little studied in any precise, experimental manner.

Problems.—This paper is the initial member in a series. The data discussed have frequently opened questions that require further analysis. The program will be well served if certain of these questions are delineated. Such delineation will aid in co-ordinating what has been said already and will also indicate general paths along which future analyses can travel. The following annotated outline focuses attention on some of these problems. This outline is illustrative rather than comprehensive.

I. Problems relating to the single-species populations
    A. *Tribolium*. The major problem raised is the oscillation cycle. Does it always occur? What are the causes? Earlier it was suggested that infection with a sporozoan, *Adelina*, may be responsible for the cycle. If this is true, an interesting opportunity exists for studying what Greenwood has called "experimental epidemiology" (see Greenwood, Hill, Topley, and Wilson, 1936). In our laboratory we have sterilized *Tribolium* of its parasites. This may furnish a tool for the analysis. In a sense such a study would be an example of interspecific competition—beetles with microörganisms.
    B. *Gnathoceros*
        1. Analysis of the "high-low" growth trend.
        2. Artificial increase of *Gnathoceros* density. It was noted that this species never builds up large populations like *Tribolium*. It would be interesting to increase the intraspecific population pressure by regularly adding new forms and observe the effect of this on the equilibrium and composition of the culture.
    C. *Trogoderma*
        1. The opportunity exists for analysis of population growth under conditions of "explosive" reproduction and short life-duration.
        2. The departure of the sex ratio in both *Gnathoceros* and *Trogoderma* from an expected 1 : 1 deserves analysis.

II. Problems relating to the mixed-species populations
    A. The effect of unequal sex ratios on population growth. This analysis would be concerned with male-female frequency as a factor in interspecific competition.
    B. More detailed analysis of "establishment" including its more precise definition, its experimental modification, and disturbance.

C. Selection experiments
  1. Selection induced by the physical environment. Varying such factors as temperature, humidity, or type of medium and assaying the effect of this on the mixed-species population.
  2. Selection primarily induced by biotic factors. Examples:
    a) The effect of cannibalism and other predation pressures on the population's growth. Cannibalism can be studied under normal (i.e., as it exists in the matings described in this paper) and artificial conditions. In the latter case the cannibalism would be manipulated in order to favor one species, etc.
    b) The effect of initial densities, different from those used here, on the competition. This problem introduces the question of wider density thresholds.
    c) Selection pressures created by artificially altering reproduction and mortality. For example, the addition of extra eggs, the removal of imagoes of certain age, etc.
    d) The possible effect of gases and secretions of beetle origin on both single and mixed-species populations.[6]

D. Migration effects. An attempt to approximate experimentally immigration and emigration by removing or adding beetles according to some logical system.

E. The effect of conditioning on population contraction. This applies to both single and mixed-species populations.

F. Analysis of possible co-operative effects either on the entire culture or one of its components.

There remains much to be said about the nature of competition pressures within populations, whether studied in the field, the laboratory, or from the viewpoint of rational mathematical analysis. The experiments reported here have described effects that are due primarily to certain aggregate behavior relationships among the beetles. Clements and Shelford (1939) have designated such relationships as "coactions"—the effect of organisms upon one another. Coactions invariably result whenever organisms come into close contact or aggregate. These coactions may take the following forms on the basis of their end effect on the population: first, they may affect adversely the physical environment (medium), in which case they are called "disoperation"; second, they may result in a more or less active demand by the population components on some substance or condition in the environment the supply of which is limited—"competition"; or, third, they may create a situation that has favorable aspects (e.g., increased survival value) for the coacting animals—"co-operation." In a recent paper Park (1941) reviewed these concepts critically and showed that many of the data of laboratory population studies can be interpreted in conjunction with this system.

The present study has described in quantitative terms certain descriptive events occurring in interspecific populations, The causes of these events are not definitely known. If they were, it would be instructive to discuss them in the light of a general ecological system such as that proposed by Clements and Shelford. Were this feasible, our data would be synthesized into a logical framework. Quantitative information would exist about the coactions that would permit their classification into descriptive, competitive, co-operative categories. It is not possible to do this now. We must await further experimental analysis. There is opportunity for much speculation at the moment, but the points still remain just that. Such factors as cannibalism and predation; aberration of metamorphosis, fecundity, and fertility; differential mortality; specific behavior patterns; competition for food and niches—all immediately suggest themselves as enticing partial explanations. At the moment we must content ourselves with the thought that this study or others like it may eventually reach such a stage. It is our opinion that ecology—and, by that token, biology—will be well served when and if such an end is attained.

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[6] Mr. Louis Roth of New York University informs us (personal communication) that he has succeeded in obtaining crystals of a secretion of *Tribolium* that cause certain abnormalities during larval development. *Gnathoceros* and *Trogoderma* may also produce analogous materials, and it is conceivable that these substances may have an effect on population, as well as on individual, growth.

As a final point it should be emphasized that, while we have discussed largely competitive coactions in this paper, excellent suggestions of co-operation also emerge from the data. For example, take the *Tribolium-Gnathoceros* case. There the ultimate success of the entire population appears to depend on the maintenance of a certain numerical density. If *Tribolium* gets too small as a culture, *Gnathoceros* is able to drive it out. Differently said, *Tribolium* is protected *as a population* by virtue of its own numbers. It is being recognized more and more that such co-operative effects grow out of aggregation and that they play major roles in species success and failure. This is true both for laboratory and for natural populations. Allee and his associates have implemented this idea frequently in the literature by presentation of evidence drawn from a catholic assembly of investigations.

SUMMARY

This investigation attempts to analyze experimentally the effect of interspecific competition on population growth. Three species of granary beetles—*Tribolium confusum*, *Gnathoceros cornutus*, and *Trogoderma versicolor*—are cultured in the same microcosm, and counts of total population size and larval, pupal, and imaginal composition are taken at regular intervals. The experiments consist of three parts: (1) control populations where each of the three species are cultured singly; (2) matings where all three species are cultured in all combinations of twos with both species seeded initially in equal density; and (3) matings where one species is introduced in numerical superiority over its competing mate. A detailed summary of major points appears in the body of the paper after each section. The following general conclusions describing the end result of the competition may be listed:

1. If *Tribolium* gets well established as a population, it drives out *Gnathoceros*.
2. If *Tribolium* populations are reduced to a low point by some type of epidemic infection (or, presumably, some other mechanism), *Gnathoceros* gets the upper hand and *Tribolium* becomes extinct.
3. *Tribolium* always drives out *Trogoderma*, irrespective of initial imaginal densities.
4. *Gnathoceros* usually drives out *Trogoderma*. This is true if the former is seeded initially in equal or higher densities. *Trogoderma* is the winner when started in a higher density than *Gnathoceros*.

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LITERATURE CITED

Allee, W. C., and Evans, Gertrude. 1937. Some effects of numbers present on the rate of cleavage and early development in *Arbacia*. Biol. Bull., 72:217–232.

Bodenheimer, F. S. 1938. Problems of animal ecology. London: Oxford Press. Pp. 183.

Chapman, R. N. 1928. The quantitative analysis of environmental factors. Ecology, 9:111–122.

———. 1933. The causes of fluctuations of populations of insects. Proc. Hawaii Ent. Soc., 8:279–292.

Clements, F. E., and Shelford, V. E. 1939. Bio-ecology. New York: John Wiley & Sons. Pp. 425.

Dick, John. 1937. Oviposition in certain Coleoptera. Ann. Applied Biol., 24:762–796.

Gause, G. F. 1934. The struggle for existence. Baltimore: Williams & Wilkins. Pp. 163.

Gause, G. F.; Smaragdova, N. P.; and Witt, A. A. 1936. Further studies on interaction between predators and prey. Jour. Anim. Ecol., 5:1–18.

Greenwood, M.; Hill, A. B.; Topley, W. W. C.; and Wilson, J. 1936. Experimental epidemiology. Medical Res. Council Rep. Ser., 209:1–204.

Johnson, W. H. 1933. Effects of population density on the rate of reproduction in *Oxytricha*. Physiol. Zoöl., 6:22–54.

Lotka, A. J. 1934. Théorie analytique des associations biologiques. Actualités scientifiques et industrielles, 187:1–45.

Nicholson, A. J. 1933. The balance of animal populations. Jour. Anim. Ecol., Suppl., 2:132–178.

Norris, Maud J. 1936. Experiments on some factors affecting fertility in *Trogoderma versicolor* Creutz. Jour. Anim. Ecol., 5:19–22.

Omori, N. 1939. Experimental studies on the cohabitation and crossing of two species of bed-bugs. Acta jap. med. trop., 1:127–154.

Park, Thomas. 1934. Observations on the general biology of the flour beetle, *Tribolium confusum* Duval. Quart. Rev. Biol., 9:36–54.

———. 1941. The laboratory population as a test of a comprehensive ecological system. *Ibid*, Part I, Vol. 16: No. 3; Part II, Vol. 16: No. 4.

Park, Thomas, and Woollcott, Nancy. 1937. Studies in population physiology. VII. The relation of environmental conditioning to the decline of *Tribolium confusum* populations. Physiol. Zoöl., 10: 197–211.

Pearl, Raymond. 1932. The influence of density of population upon egg production in *Drosophila melanogaster*. Jour. Exper. Zoöl., 63:57–111.

Reich, Karl. 1936. Studies on the physiology of *Amoeba*. I. The relation between nutrient solution, zone of growth, and density of population. Physiol. Zoöl., 9:254–263.

Smaragdova, N. P. 1936. The interaction between phyrophagous and predatory mites in relation to their environment and concentration. Bull. Biol. and Med. Exper., 2:99–100.

Vladimerova, M. S., and Smirnov, E. S. 1938. The struggle for food in homogeneous and in mixed populations of *Musca domestica* and *Phormia groenlandica*. Meditsinskaia parazitologiia i parazitarnye bolezni, 7:755–777.

Volterra, Vito. 1928. Variations and fluctuations of the number of individuals in animal species living together. Jour. du conseil internat. pour l'exploration de la mer, Vol. 3. Translated in Chapman, Animal Ecology (1931), pp. 409–448.

GROWTH STUDIES OF COLPODA DUODENARIA. I. STERILIZATION OF THE CILIATES

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RECENT investigations of Taylor, Brown, and Strickland (1936) and of Taylor and Strickland (1938, 1939) relating factors of the environment to the reactivities of *Colpoda duodenaria*, which involve its protoplasmic reorganization, have shown that it is desirable to standarize this experimental material. This follows from the general assumption that the developmental stage of the cell or oganism, as well as its genetic and environmental history, are indispensable components of the conditions necessary for reproducible results.

The first step in standardization is the cultivation of *C. duodenaria* in purely synthetic or at least well-defined and reproducible media. These organisms are grown in a balanced inorganic salt medium with no organic constituents other than the suspension of bacteria (a pure culture of a selected *Pseudomonas* sp. [?]) which is added as required for food. It is evident that this is by no means a defined medium. It was our aim to cultivate the *Colpoda* free from any other living organism.

Various methods have been described for the sterilization of Protozoa. Oehler (1919, 1920, 1924) was the first to report a bacteria-free culture of a ciliate. He obtained sterile individuals of *C. steinii* by isolating those that had migrated over the surface of an agar plate. He also obtained sterile cultures of *C. cucullus* by the same technique. Lwoff (1924) reported some data on cultivation of sterile *Glaucoma piriformis* but gave no details about the method used in the sterilization of this protozoan. Parpart (1928) sterilized *Paramecium caudatum* by means of repeated transfers of a single animal through sterile broth media. Peters (1929) claimed to have grown *C. colpoda* in bacteria-free cultures with inorganic nitrogen sources. However, his cultures actually contained bacteria (Gatenby and Cowdry, 1928). Butterfield (1929, 1933) obtained *Colpidium* (*G. piriformis*) in a sterile culture. Luck and Sheets (1931) made extensive efforts to sterilize cysts of *Euplotes taylori* by means of chemical reagents, but without success. The cysts were found to be less resistant to disinfectants than were the associated bacteria. Hetherington (1933) sterilized *C. campylum*, *G. scintillans*, and *Loxocephalus granulosus* by the washing method of Parpart. Elliott (1933) obtained *C. striatum* in a bacteria-free culture but did not give any details about the method used. In a recent paper Brown (1940) sterilized *Leucophrys patula* and *G. piriformis* by individual washing and migration through dilute agar (0.5 per cent or less).

By all these methods of sterilization it is possible to obtain only a few sterile ciliates at a time. It was desirable for our purposes to sterilize as many animals as possible in one treatment. This report will deal with attempts made to find such a method.

METHODS OF STERILIZATION

I. THE CENTRIFUGING METHOD

Kidder and Stuart (1939) described a simple method to get approximately 100 sterile *Colpoda* at one time. These were obtained by centrifuging and with much dilution of the *Colpoda* culture. This procedure was followed in essentials. Large numbers of *Colpoda* cysts[1] were excysted in 10 cc. sterile yeast extract (1.5 per cent Difco yeast). As soon as excystment started, the yeast extract was replaced by 10 cc. sterile balanced salt medium.[2] After complete excystment the solution, containing the free-swimming organisms, was transferred by means of sterile pipette to a sterile centrifuge tube (15 cc.), which was stoppered with a cloth-covered cotton plug. The ciliates were concentrated by slow centrifugation (1,200 r.p.m. during 5 minutes). Then 9 cc. of the solution were withdrawn and the tube was allowed to stand for about 5 minutes so that the ciliates might swim to the top of the remaining cubic centimeter of solution. With a sterile pipette 0.9 cc. of the ciliate suspension was withdrawn and placed in an empty sterile centrifuge tube. This suspension was diluted to 10 cc. with sterile distilled water, and the whole process was repeated until the ciliates had gone through twenty such transfers with the accompanying dilutions. This method entails a great loss of ciliates, and it failed to sterilize *C. duodenaria*. After innoculation of the ciliates thus washed twenty times into a yeast-glucose broth (1.5 per cent Difco yeast, 2 per cent glucose), prompt development of bacteria took place.

II. HEAT TREATMENT

Taylor and Strickland (1936) suggested that cysts of ciliates might be sterilized by exposure of dry cysts to high temperatures or to a high vacuum. This suggestion was followed, but without success. In brief, the procedure was as follows: a culture of *C. duodenaria* was centrifuged and washed three times under the conditions previously described. About 1 cc. of the concentrated suspension was placed in a Stender preparation dish. A sheet of cellophane was placed between the cover and the dish. Top and bottom were held together with a tight rubber band. The whole unit was autoclaved in a high Petri dish for 15 minutes at 130° C. After introduction of the ciliates the dish was inverted. The ciliates encysted on the cellophane. After complete encystment the sheet of cellophane was rinsed several times with sterile distilled water and dried under reduced pressure over P_2O_5. The sheet was then cut in small strips with sterile precautions and each strip placed in a sterile culture tube. These tubes were heated at different temperatures for lengths of time varying from 1 to 10 hours. After the heating, 2 cc.

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[1] In all our experiments *C. duodenaria* were grown in sterile Petri dishes in order to avoid any air contamination.

[2] The balanced salt medium had the following composition:

Distilled water	100 cc.
NaCl	0 0104 gm.
$MgCl_2\ 6H_2O$	0.0085
$MgSO_4\ 7H_2O$	0.004
KCl	0.0023
$CaCl_2$ anh.	0.001

All chemicals were obtained from Merck quality reagent.

of the yeast-glucose medium were added to each tube. After complete excystment, 0.1 cc. of this excystment medium containing *Colpoda* was placed in a tube with yeast-glucose broth, and 0.1 cc. was plated on yeast-glucose agar.[3] As can be seen in Table 1 (showing only the results of the 10-hour heat treatment), the outcome of these experiments was discouraging. Not only were the results irregular (e.g., at 55° and at 65°), but heating of the dry cysts at 65° for 10 hours killed about 80 per cent of the cysts, while bacterial growth was visible after 3 days. Intermittent heat treatment did not solve the problem.

TABLE 1

TEMPERATURE (°C.)	EXCYSTMENT*	BACTERIAL GROWTH†			
		1 Day	2 Days	3 Days	4 Days
40	+++++	....	....	250	....
45	+++++	....	15	....	......
50	+++++	....	......	130	......
55	++++	......	.....	......	25
60	+++	......	....	91	....
65	+	......	....	115	......

* Each + stands for 20 per cent excystment

† Given in number of colonies on the agar plates The tubes with yeast-glucose broth showed growth of bacteria on the same day.

Dry cysts were heated for 3 hours, excysted, and after centrifuging and subsequent washing (see Sec. I) were again made to encyst. These resulting cysts were killed by a second heating at 60° for 3 hours.

III. HIGH VACUUM TREATMENT

Heating at 60° and at a vacuum of 10^{-6} mm. Hg in a vacuum desiccator (after Abderhalden) for 4 hours and for 10 hours did not sterilize the cysts. In the first case bacterial growth was evident after 24 hours; in the latter after 48 hours. Excystment in the second case was diminished to 50 per cent.

IV. BACTERIOPHAGE

Broth media can be kept sterile by introducing with the bacteria a bacteriophage solution specific for that particular strain of bacteria. It seemed possible that a suitable bacteriophage would autolyze the bacteria and not affect the ciliates. It was found that the *Colpoda* were not affected by the presence of the phage but also that the phage failed to autolyze all the bacteria. Since bacteriophage affects bacteria only in the early growing stage, the technique finally used was as follows: *C. duodenaria* were cultured in the usual way. Instead of the *Pseudomonas* sp., *Listerella monocytogenes* (Schultz strain)

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[3] Autoclaved 15 minutes at 120° C.

Difco yeast	7.5 gm.
Glucose	10
Agar (Difco)	10
Distilled water	500

(*L. monocytogenes*, K 12)[4] were given as food. After encystment the cysts were washed several times with sterile distilled water. Yeast-glucose broth was added and at the same time 0.2–0.5 cc. of the *L. monocytogenes* phage solution. Excystment was normal within 4 hours. The tubes stayed clear for 24 hours and in some cases even for 48 hours. The *Colpoda* multiplied during this period. But in all cases bacterial growth was visible within 48 hours. The control tubes (inoculated only with *L. monocytogenes* and the phage solution) remained clear and free from bacteria in all cases. There seem to be two possibilities: (1) *C. duodenaria* excrete a substance which inhibits the lysing action of the bacteriophage. (2) Some bacteria are not digested and become phage resistant by passing through the food vacuoles.

Since *L. monocytogenes* easily forms phage-resistant strains, the same experiments were repeated with two strains of *Staphylococcus aureus: Staph. aureus* AD 8 and *Staph. aureus* AD 11 together with the bacteriophage specific for these strains, Phage K 115.[5] The results, however, were the same as those obtained with *L. monocytogenes*. An intermittent addition of phage solution was also unsuccessful. Cysts were treated with yeast-glucose broth with additional phage solution. After complete excystment the solution was centrifuged and the animals washed three times (see Sec. I). The *Colpoda* were then placed in a fresh yeast-glucose broth with 0.5 cc. phage solution. After 3 hours the whole procedure was repeated, and 3 hours later it was repeated again. In this last tube bacterial growth again took place within 48 hours.

TABLE 2

Time in Minutes	Excystment	Bacteria
2.......	Normal	Growth within 48 hours
4.......	Normal	Growth within 48 hours
8.......	Normal	Growth within 48 hours
10	Normal	Growth within 48 hours
14.......	Delayed	Growth within 48 hours
18.......	Delayed, 50 per cent excystment	Growth within 48 hours
20.......	No excystment	Sterile

V. ULTRAVIOLET IRRADIATION

Giese and Leighton (1935) studied the influence of ultraviolet radiation upon *Paramecia*. *Paramecia* did not die when irradiated with high dosages of intense light of wave length 3130 Å. By using shorter wave lengths, vesiculation occurred when between 10^{12} and 10^{13} quanta had been adsorbed by a *Paramecium*.

For the following tests a mercury lamp, type H_4 (G.E.), was used. The *C. duodenaria* were cultivated in quartz dishes. After encystment the cysts were washed several times with sterile distilled water and irradiated in the wet state. An irradiation time of 20 minutes (Table 2) killed both *Colpoda* and bacteria, while an irradiation of 18 minutes delayed and decreased the excystment and apparently did not injure the bacteria.

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[4] We obtained *L. monocytogenes* K 12 and the phage K 12 from Professor C. E. Clifton, for which acknowledgment is hereby gratefully made.

[5] These were also obtained from Professor C. E. Clifton.

VI. PARPART'S METHOD (MODIFIED)

Raper (1937) has published a method of freeing fungi from bacterial contamination. He used a 2 per cent agar medium and let the fungi grow through the agar.

Combining this method with Parpart's washing method produced excellent results. Instead of the sterile water drops used by Parpart, sterile drops of a 0.5 per cent agar solution were used. After migrating through 3 drops, each having a diameter of approximately 3 mm., single individuals were transferred to a yeast-glucose broth. The tubes did not show any bacterial growth even after 7 weeks' incubation. During this period 0.1 cc. of the broth was plated out several times and 0.1 cc. transferred to fresh broth, but no bacterial growth could be observed. The *Colpoda*, however, did not multiply. It is therefore possible to sterilize *C. duodenaria* by this technique, but only one ciliate at a time could thus be obtained. Therefore, an attempt was made to utilize this principle of migration through agar in obtaining large numbers of sterile *Colpoda*. Since *C. duodenaria* always tend to swim to the surface of the medium, these ciliates were introduced at the bottom of an agar column in a glass tube. They migrated to the surface of the agar. During this passage the bacteria on the surface of the *Colpoda* were scraped off. Bacteria which were in the food vacuoles were supposedly digested by the *Colpoda* during the migration through the agar solution.

The technique which finally proved to be the most satisfactory and which made possible the sterilization of as many as 50 animals in one treatment was as follows: a glass tube of 15-cm. length and 7-mm. diameter was closed at one end with a serum cap and filled with a 0.35 per cent agar solution. The agar column was 75 mm. long. The tube was closed with a cotton plug and sterilized in the autoclave. A concentrated culture of *C. duodenaria* was centrifuged and washed three times as described before. The concentrate was then centrifuged through a sterile agar solution (0.35 per cent) in a 15-cc. centrifuge tube. This was done at such a speed that most of the *Colpoda* were thrown down in about 2 hours. The upper part of the agar was withdrawn by means of a sterile pipette. The remaining 0.5 cc. was injected with a sterile syringe through the serum cap. The *Colpoda* were then at the bottom of the agar column. The tube was left in a vertical position for 6–7 hours. At the end of that time enough *Colpoda* had migrated to the surface of the agar column to be removed. This was done by means of a sterile micropipette. The *Colpoda* were transferred to tubes containing yeast-glucose broth and were observed for 7 weeks. Frequently during that time 0.1 cc. was plated onto yeast-glucose agar and also 0.1 cc. innoculated into fresh yeast-glucose broth to test for bacteria. No bacterial growth was observed during the 7 weeks, and no bacteria could be detected on microscopical examination of the broth cultures. Therefore it may be assumed that these *Colpoda* were sterile.

SUMMARY

Several methods, described in the literature for sterilizing ciliates, proved unsatisfactory for the sterilizing of *C. duodenaria*. A new method is described which involves migration of *Colpoda* through a sterile viscous agar medium. The *C. duodenaria* so obtained were rigorously tested for sterility during 7 weeks after the migration through the agar and proved to be sterile.

LITERATURE CITED

Brown, M. G. 1940. Growth of protozoan cultures. II. *Leucophrys patula* and *Glaucoma piriformis* in a bacteria-free medium. Physiol. Zoöl., 13:277.

Butterfield, C. T. 1929. III. A note on the relation between food concentration in liquid media and bacterial growth. U.S. Public Health Repts., 44:2865.

———. 1933. A note on the food habits of *Colpidium*. *Ibid.*, 48:814.

ELLIOTT, A. M. 1933. Isolation of *Colpidium striatum* Stokes in bacteria-free media. Biol. Bull., **65**:45.

GATENBY, J. B., and COWDRY, E. V. 1928. Bolles Lee's Microtomist's Vade mecum. Philadelphia: Blakiston.

GIESE, A. C., and LEIGHTON, P. A. 1935. Quantitative studies on the photolethal effect of quartz ultraviolet radiation upon *Paramecium*. Jour. Gen. Physiol., **18**:557.

HETHERINGTON, A. 1933. The culture of some holotrichous ciliates. Arch. f. Protistenk., **80**:255.

KIDDER, G. W., and STUART, C. A. 1939. Growth studies on ciliates. I. The role of bacteria in the growth and reproduction of *Colpoda*. Physiol. Zoöl., **12**:329.

LUCK, J. M., and SHEETS, G. 1931. The sterilization of Protozoa. Arch. f. Protistenk., **90**:396.

LWOFF, A. 1924. Le Pouvoir de synthèse d'un protiste hétérotrophe: *Glaucoma piriformis*. Compt. rend. Soc. biol., **91**:344.

OEHLER, R. 1919. Flagellaten und Ciliatenzucht auf reinem Boden. Arch. f. Protistenk., **40**:16.

———. 1920. Gereinigte Ciliatenzucht. *Ibid.*, **41**:16.

———. 1924. Gereinigte Zucht von freilebenden Amöben, Flagellaten und Ciliaten. *Ibid.*, **49**:287.

PARPART, A. K. 1928. The bacteriological sterilization of *Paramecium*. Biol. Bull., **55**:113.

PETERS, R. A. 1929. Observation upon the oxygen consumption of *C. colpoda*. Jour. Physiol., **68**:1.

RAPER, J. R. 1937. A method of freeing fungi from bacterial contamination. Science, **85**:342.

TAYLOR, C. V., BROWN, M. G., and STRICKLAND, A. G. R. 1936. Effects of a given X-ray dose on cysts of *Colpoda steinii* at successive stages of their induced excystment. Jour. Cell. and Comp. Physiol., **9**:105.

TAYLOR, C. V., and STRICKLAND, A. G. R. 1936. Effects of high vacua and extreme temperatures on cysts of *Colpoda cucullus*. Physiol. Zoöl., **9**:15.

———. 1938. Reactions of *Colpoda duodenaria* to environmental factors. I. Some factors influencing growth and encystment. Arch. f. Protistenk., **90**:396.

1939. Reactions of *Colpoda duodenaria* to environmental factors. II. Factors influencing the formation of resting cysts. Physiol. Zoöl., **12**:219.

EXPERIMENTAL STUDIES ON A JAPANESE PLANARIAN
II. AXIAL DIFFERENTIAL IN RATE OF EYE FORMATION IN RECONSTITUTION

(Three figures)

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IN AN earlier paper by Child (1932) it was shown that a Japanese triclad known as *Planaria gonocephala*[1] resembles two American species, *Dugesia* (= *Euplanaria*) *dorotocephala* and *D. tigrina* (= *E. maculata*)[2] in possessing a definite fission zone and a physiologically well-defined posterior zooid region and in showing differential susceptibility along the longitudinal axis of the body to toxic concentrations of various agents used. In these three species the susceptibility is highest at the anterior end and decreases posteriorly to the level of fission but increases more or less again in the posterior zooid region. In the two American species the data on head frequency—i.e., capacity for and degree of head reconstitution—agree with those on susceptibility to lethal agents not only in indicating the existence of an axial physiological differential but also as regards its general form (Child and Watanabe, 1935; Watanabe, 1935*b*; and citations). In the present species, as has been pointed out (Watanabe, 1937, p. 294, n.), head frequency is extremely high and even 1/16 pieces from any level all develop normal heads. Consequently, no axial differential can be detected. However, it would be premature to deny the existence of an axial differential in the activity of reconstitution in this species on the basis of this evidence, because, besides head frequency, there is another important factor in head development, namely, rate of differentiation which may be regarded as representing an intensity factor in certain reconstitutional processes.

Data on the axial differential in rate of head development as indicated by time of appearance of eyespots have been recently given for several species of single-zooid planarians.[3] But, as to the planarians which undergo fission and have a distinct posterior zooid region, the data at hand are for only the one species, *D. dorotocephala* (Watanabe, 1935*a*). In pieces of given length of this species eyespots appear the later, the more posterior the level of head formation within the anterior zooid. Also heads with abnormal eyes (connected eyes and single median eyes), which appear in shorter pieces from the more posterior pieces of the anterior zooid, develop less rapidly than those with normal symmetrical eyes. Evidently, rate of eye formation and head frequency are associated in *D.*

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[1] Although this species is called *Planaria gonocephala*, it differs widely as regards physiological characteristics from the European *P. gonocephala* (Abeloos, 1930) and is probably a different species.

[2] According to Hyman (1931), American species of the genus earlier called *Planaria* must be called *Euplanaria*. More recently (Hyman, 1939) she has stated that the genus name *Dugesia* has priority over *Euplanaria*. Since the genus is closely related to the European *Planaria* and members of the group have long been known as planarians, *Euplanaria* is a far more satisfactory designation. The result of strict application of the rule of priority seems particularly unfortunate in this case.

[3] *Planaria gonocephala* (Abeloos, 1927, 1930), *Planaria lugubris* (Sivičkis, 1933, 1934), *Dendrocoelum lacteum* (Sivičkis, 1931*b*), *Polycelis nigra* (Sivičkis, 1933), *Phagocata gracilis* (Buchanan, 1933).

dorotocephala. For this reason it was thought desirable to work with other planarians in which the head-frequency factor can be eliminated even in short pieces. In this respect the Japanese species provides very favorable material. The present investigation is, then, concerned with the question of the relation of rate of head development to level of body and length of piece as indicated by time of appearance of eyespots and quite independently of head frequency. To Dr. C. M. Child, of Stanford University, thanks are due for his constant encouragement and helpful suggestions.

MATERIAL AND METHOD

The worms were collected from spring-fed streams in and about the city of Sapporo. For the experiment animals which had not recently undergone fission were selected and were kept without food 5 days before experiment. In most cases lots of 20 pieces each were used, and the experiment was repeated to a total of 200 pieces. Pieces were cut transversely with a small scalpel, and each 20 pieces from the same body-level were placed together in 200 cc. of fresh well water—i.e., 10 cc. of water for each piece—and kept in a dark cabinet without change of water. The temperature in the cabinet was controlled by thermostat. Temperature variation was not over 3.0° C., in each case.

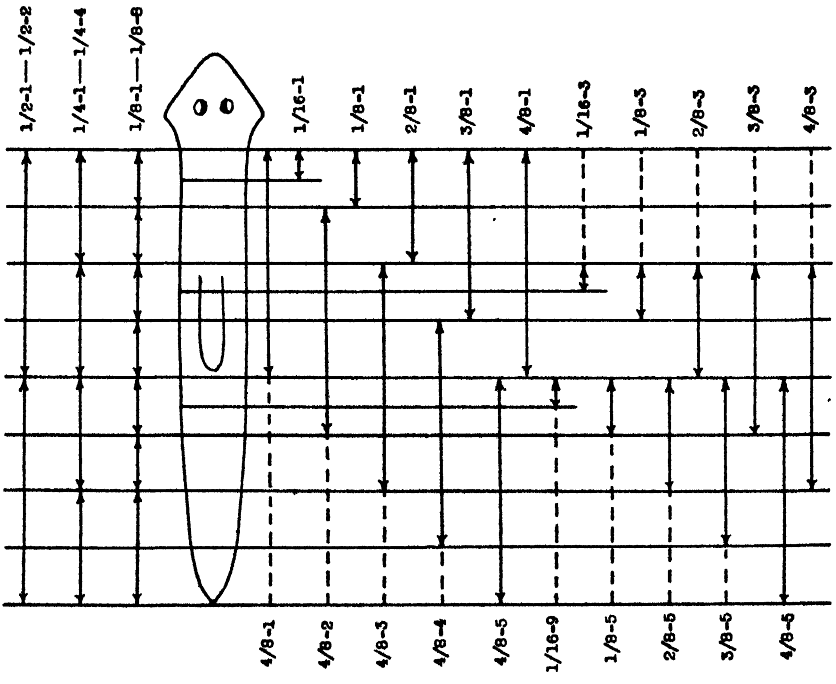


FIG. 1.—Diagram showing pieces, lengths, and levels of body used

Determination of presence or absence of developed eyespots was made at 8- or 12-hour intervals with compound microscope, magnification of fifty, and careful focusing below the epithelium; eyespots were distinguishable from the body pigment by their deeper location. From the numbers of pieces with eyespots thus recorded the arithmetical means of time from section to appearance and average standard deviations within lots were calculated. The statistical probability (P) that as great a difference might be given by random sampling was also calculated with use of "Students' " method and a table of t (Fisher and Yates, 1938).

The pieces of equal length from different levels of the same bodies are indicated numerically according to the fraction of the postcephalic length which the pieces represent—1/2, 1/4, 1/8—and the body-level by a numeral following the fraction, as 1/2-1 to 1/2-2, 1/4-1 to 1/4-4, and 1/8-1 to 1/8-8, the level 1 being the anterior level just behind the head. Pieces of different lengths with anterior ends at the same level are designated as follows: 1/16-1, 1/8-1, 2/8-1 to 4/8-1; 1/16-5, 1/8-3, 2/8-3 to 4/8-3; 1/16-9, 1/8-5, 2/8-5 to 4/8-5, the numeral following the fraction indicating the level of the anterior cut end of the piece. Pieces of equal length from different levels but so long that they are of necessity from different animals—e.g., 4/8 pieces—are designated 4/8-1, 4/8-2 to 4/8-5. The various pieces, lengths, and levels used are indicated in Figure 1.

EXPERIMENTAL RESULTS

Times of appearance of eyespots in pieces of equal length from different levels of the same animals.—In these experiments the mean times of appearance of eyespots of 1/2, 1/4, and 1/8 pieces were determined in animals of 9–10 mm. and 14–15 mm. in length. The material of the two different lengths was collected at different times of year.

As the data given in Table 1 show, the mean time of appearance of eyespots is least at the most anterior level and increases to the middle region but decreases again at more posterior levels. In other words, the rate of eye formation is highest at the most anterior level and decreases toward the posterior end of the anterior zooid but again increases in the posterior zooid region (cf. the last column of Table 1). The values of statistical probability, P, for difference of average mean times between successive levels indicate high significance in the anterior levels and less significance in the middle levels. Such anteroposterior variation in P is undoubtedly dependent on the greater variation in mean times of the more posterior pieces from the anterior zooid. In each series, with some exceptions, average standard deviation of mean times increases also from anterior to posterior levels of the anterior zooid and decreases again in the posterior zooid region.

Rate of eye formation of 1/8 pieces from an abnormal stock.—Even though the reconstitution of pieces takes place at the same temperature, the rate of eye formation shows some variations with different stocks of worms collected at different times of year, from different localities, and kept for different lengths of time in the laboratory. Nevertheless, the axial relation of rate to level of body is usually similar to that described above. However, the rates in certain worms collected from one locality in midsummer of 1939 were almost identical at all levels of body. The mean times and rates of 1/8 pieces from these worms of 14–15 mm. in length are shown in Table 2.

The animals of this stock were apparently, for some unknown reason, not in normal condition. One month after the collection many individuals began to disintegrate, and during the following two weeks all died, though stocks usually live indefinitely under laboratory conditions. These anomalies apparently represented a temporary condition, because worms from other collections from the same spring-fed stream did not show them.

Effect of length of piece on rate of eye formation.—The data in Table 1 suggests that the rate at a given level decreases with decrease in piece length. In order to obtain more ac-

TABLE 1

MEAN TIMES IN HOURS TO APPEARANCE OF EYES IN 1/2, 1/4, AND 1/8 PIECES

Data are averages of ten lots, each of pieces from 20 worms, a total of 200 worms. Examinations were made at 8-hour intervals.

Body Length (In Mm.)	Temperature Range (° C.)	Pieces	Average Mean Times	Average Standard Deviations	P	Relative Rate in Percentages
9–10	27–30	1/2-1	48.9	4.97	<0.001	100.0
		1/2-2	54.2	7.09		90.3
		1/4-1	49.6	5.34	<0.001	100.0
		1/4-2	54.2	6.70	0.002	91.3
		1/4-3	55.7	7.01	0.018	89.4
		1/4-4	54.9	6.89		90.7
		1/8-1	48.0	4.37	<0.001	100.0
		1/8-2	50.1	5.48	<0.001	95.8
		1/8-3	53.6	6.94	0.006	89.6
		1/8-4	55.5	6.92	0.168	86.5
		1/8-5	56.2	7.15	0.011	85.4
		1/8-6	58.1	6.62	0.803	88.8
		1/8-7	53.9	7.10	0.061	89.1
		1/8-8	52.8	5.78		90.9
14–15	27–30	1/2-1	49.1	5.04	<0.001	100.0
		1/2-2	55.7	6.58		88.4
		1/4-1	50.6	6.70	<0.001	100.0
		1/4-2	56.0	8.62	<0.001	90.3
		1/4-3	60.1	8.85	<0.001	84.3
		1/4-4	58.2	8.93		87.0
	25–28	1/8-1	50.6	6.29	0.005	100.0
		1/8-2	53.0	7.00	<0.001	95.5
		1/8-3	56.9	7.30	0.006	89.3
		1/8-4	59.4	7.42	0.465	85.3
		1/8-5	60.2	7.77	0.003	84.1
		1/8-6	57.0	7.59	0.064	88.7
		1/8-7	56.0	8.25	0.055	90.3
		1/8-8	55.0	7.95		92.0

curate experimental evidence concerning the relation between rate of eye formation and length of piece, the mean times of pieces of five lengths—1/16, 1/8, 2/8, 3/8, and 4/8—with the anterior ends at the same levels (Fig. 1) were determined, and from these means the rates of the pieces relative to 1/8 pieces from the same individuals were calculated, except at the level 1, where the relative rates were determined as percentages of the rates of 1/8 pieces from all other individuals of the same lots. Pieces with anterior ends at the same levels came from the same stock, but those for different levels from different stocks. Consequently, only the data for the pieces from the same levels are directly comparable.

The data in Table 3 show, first, that, at the anterior level immediately behind the head, rates of eye formation are nearly equal for pieces of different lengths and, second, that at the more posterior levels there is in general a continuous increase with increase in length of piece from 1/16 to 3/8 and no appreciable increase in rate from 3/8 to 4/8. The values of P indicate that, at the level just behind the head, differences for the pieces of increasing length are not significant, but at the posterior levels of the anterior zooid those for 1/16-5 and 1/8-3, 1/8-3 and 2/8-3, 2/8-3 and 3/8-3, 1/16-9 and 1/8-5, 1/8-5 and 2/8-5 are all significantly different. As shown in the last column of the table, average standard deviation, with a few exceptions, decreases as the length of piece increases at the posterior levels, while it does not appreciably change at the most anterior level.

TABLE 2

MEAN TIMES IN HOURS TO APPEARANCE OF EYES IN 1/8 PIECES FROM AN ABERRANT STOCK

Data are averages of ten lots, each consisting of pieces from 20 worms of 14–15 mm. in length: 200 worms; temperature range: 27°–30° C. Examinations at 8-hour intervals.

Pieces	Average Mean Times	Average Standard Deviations	P	Relative Rate in Percentages
1/8-1.	45 9	2 49		100 0
			0 017	
1/8-2	46 7	3 31		98 3
			0 001	
1/8-3.	48 5	4.42		94 6
			0 190	
1/8-4...	47 9	4 38		95 8
			0 336	
1/8-5.	47 7	4 65		96 2
			0 144	
1/8-6........	47 0	3 52		97 7
			0 097	
1/8-7........	46 0	3.24		99 8
			<0 001	
1/8-8...... ..	47 3	3 40		97 0

Effect of posterior half-section on rate of eye formation.—These experiments are supplementary to the preceding. As shown above, in posterior pieces from the anterior zooid the rate of eye formation decreases with decrease in length of piece. If this is due to the effect of length of piece or to a stimulation from the posterior section, it should be possible to retard the rate of eye formation on one side of the piece by making a posterior half-section a short distance posterior to the anterior section. The following experiments were undertaken in order to test this possibility.

In 1/2 pieces from animals 18–20 mm. in length a transverse section was made from one side of the body to the median line 1.5 mm. posterior to the anterior section (Fig. 2), or a strip of tissue half the width of the body was removed posterior to this half-section (Fig. 3). Pieces used for a single half-section and those for removal of tissue came from different stocks. Results are given in Table 4.

TABLE 3

RATE OF EYE FORMATION AT THREE DIFFERENT LEVELS OF BODY IN RELATION TO LENGTH OF PIECE

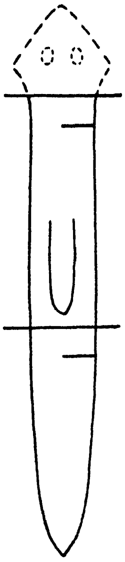
Data are averages of ten lots, each of 20 pieces, for each length from 12–13 mm. worms, i.e., 1,000 worms for each level. Examinations at 8-hour intervals.

Temperature (° C.)	Pieces	Relative Rate in Percentages	P	Average Standard Deviations (In Hours)
24–27	1/16-1	100.1		11.5
			>0.900	
	1/8-1	100.0		11.7
			0.404	
	2/8-1	99.4		11.6
			>0.900	
	3/8-1	99.5		11.8
			0.369	
	4/8-1	99.9		11.6
19–22	1/16-5	81.6		14.2
			0.004	
	1/8-3	86.5		14.2
			0.038	
	2/8-3	89.6		13.3
			0.042	
	3/8-3	92.8		13.6
			0.500	
	4/8-4	93.0		13.0
	1/16-9	77.8		12.1
			<0.001	
	1/8-5	84.5		11.9
			0.021	
	2/8-5	87.6		11.9
			0.161	
	3/8-5	90.0		10.6
			0.271	
	4/8-5	90.8		10.1

In anterior 1/2 pieces, with or without removal of tissue, average mean times of appearance of eyespots are slightly longer on the side of half-section than on the intact half, but the differences in time are less than 1 hour and entirely without significance as indicated by high values of statistical probability, P. In posterior 1/2 pieces average mean times of the side of half-section are 5–7 hours longer than those of the other side.

Though such differences seem to be rather small as compared with the mean times, the values of P for these differences indicate that they are unquestionably highly significant. That is to say, at the anterior level immediately posterior to the head the half-section does not affect the rate of eye formation, but at the posterior level a retardation of eye formation is clearly seen on the side of the half-section.

In this series the number of pieces which developed one eyespot on one side of the piece but none on the other was recorded at the time of examination of pieces. Percentages of such pieces are appended in the last two columns of Table 4: the column headed "Asymmetry" indicates cases in which the eyespot developed later on the side of half-section than on the side without half-section, and the column "Reversed Asymmetry," cases in which the eyespot developed earlier on the side of half-section than on the other. These percentages show also a distinct retardation of eye formation on the side of half-section at the posterior level.



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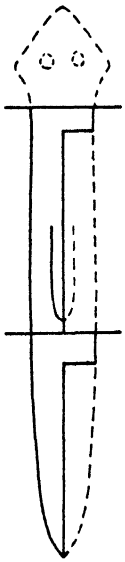

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FIG. 2.—Posterior half-sections at anterior and posterior levels, without removal of tissue.

FIG. 3.—Posterior half-sections at anterior and posterior levels, with removal of tissue.

Effect of posterior half-section upon the symmetry of head forms.—Retardation of development on the side of half-section makes the new head temporarily asymmetrical not only in time of appearance of eyespots but also in size of developed eyespots, amount of new tissue, and shape of lateral lobes (cf. Child and Watanabe, 1935, Figs. 12–29). For more precise determination of the percentages of symmetrical, asymmetrical, and reversed asymmetrical heads, another set of 1/2 pieces was prepared with the same procedures as in the preceding series and was examined at much shorter time intervals (less than 6 hours). Table 5 gives the results.

Table 5 shows that both with and without removal of tissue 64 and 65 per cent of heads at anterior level are symmetrical in all respects. At the posterior level, however, development is retarded on the side of half-section in 64 and 66 per cent. Reversed asymmetry occurs in 16 and 20 per cent at the anterior level, and in 7 and 10 per cent at the posterior level. Statistical probability, P, for difference in percentages between asymmetry and reversed asymmetry at the anterior level is 0.212 without removal of tissue and 0.209 with removal of tissue. These differences have little significance. Consequently, occurrence of asymmetry and reversed asymmetry at the anterior level is probably due to incidental factors having no relation to the half-section. At the posterior level, however, the values of P for the difference between asymmetry and reversed asymmetry are less than 0.001, that is, highly significant. All these data make it clear that at the posterior level a half-section and removal of a strip half the width of the body posterior to the half-section retard the development on the side of the injury but are not effective at the anterior level just behind the head.

Rates of eye formation in 4/8 pieces from different levels.—The preceding series have shown that the rate of eye formation varies with length of piece as well as with level of body. In the present series the rates of 4/8 pieces from five different levels of body were determined in order to eliminate length of piece as a factor in rate of eye formation and to show whether an axial differential independent of relation to piece length is present. The five levels of 4/8 pieces used differ by 1/8 of the postcephalic length (Fig. 1). The 4/8 pieces were necessarily taken from different individuals, but the mean times were also determined for 1/8-1 pieces of the same individuals, except in the case of 4/8-1 pieces where this is impossible, and the rates were calculated relative to these 1/8-1 pieces as a standard. For 4/8-1 pieces the rates were calculated from the mean times of 4/8-1 pieces and averages of four mean times of 1/8-1 pieces from individuals used for 4/8-2 to 4/8-5 pieces of the same lots. With these long pieces less difference in rate between anterior 4/8 and posterior 4/8 pieces was expected than with shorter pieces. For this experiment a stock was selected by preliminary examination showing a relatively

TABLE 4

RATE OF EYE FORMATION IN 1/2 PIECES AFTER POSTERIOR HALF-SECTION WITH AND WITHOUT REMOVAL OF TISSUE

Data are averages of ten lots, each of pieces from worms 18–20 mm. in length. Examinations at 12-hour intervals. Temperature: 9°–10° C.

Operation	Pieces	Average Mean Times in Hours		Difference		Asymmetry in Percentages	Reversed Asymmetry in Percentages
		Half-cut side	Intact side	In Hours	P		
Without removal of tissue	1/2-1	123.0	123.6	0.6	0.726	9	84
	1/2-2	134.6	139.6	5.0	0.020	35	
With removal of tissue	1/2-1	126.1	126.9	0.8	0.9	15	7
	1/2-2	144.6	151.6	7.0	0.008	55	4

TABLE 5

HEAD FORMS DEVELOPED (IN PERCENTAGES) AFTER POSTERIOR HALF-SECTION

Data are averages of five lots, each consisting of 20 pieces, from worms 18–20 mm. in length. Temperature: 12°–14° C.

Operation	Pieces	Symmetry	Asymmetry	Reversed Asymmetry
Without removal of tissue..	1/2-1	65	15	20
	1/2-2	26	64	10
With removal of tissue....	1/2-1	64	20	16
	1/2-2	27	66	7

great difference in rate between pieces 4/8-1 and 4/8-5.[4] In Table 6 average rates, values of P, and average standard deviations obtained are given.

From the data of Table 6 it is clearly seen that average rates show a continuous decrease from the anterior to the posterior level. The values of P indicate, however, that the differences for the first and second and for the second and third 4/8 pieces are clearly significant, while those for the third and fourth and for the fourth and fifth 4/8 pieces are less significant. Average standard deviation of mean times increases from anterior to posterior levels of anterior zooid, with an exception in 4/8-5 pieces.

TABLE 6

RATE OF EYE FORMATION IN 4/8 PIECES FROM FIVE DIFFERENT BODY-LEVELS

Data are averages of ten lots, each consisting of 20 pieces for each level from worms 13–14 mm. in length: total, 1,000 worms. Temperature: 16°–19° C. Examinations at 12-hour intervals.

Pieces	Relative Rate in Percentages	P	Average Standard Deviations (In Hours)
4/8-1. . . .	99.9		13.2
		<0.001	
4/8-2. . . .	94.8		17.1
		0.021	
4/8-3. . . .	89.9		19.3
		0.080	
4/8-4	86.9		21.2
		0.085	
4/8-5.	84.5		20.0

DISCUSSION

In considering the data presented above it is seen that the mean times of appearance of eyespots and the relative rates as percentages of a mean time taken as representing a standard rate differ in definite and orderly ways with level of body and length of piece. As noted in an earlier paper (Watanabe, 1935*a*), the relative rates do not involve any assumptions as to constancy of rate from section to appearance of eyespots but merely show certain relations of mean times and indicate only difference in rate of a series of changes which occur between section and appearance.

The data of Table 1 indicate that the rate of eye formation decreases in general from anterior to posterior levels of the anterior zooid and increases at more posterior levels. But the posterior rise is not always statistically significant. This axial relation of rate is similar to that of head frequency (Child and Watanabe, 1935) and of the eye formation (Watanabe, 1935*a*) in *D. dorotocephala*. It appears, then, that the rate of eye formation as an indicator of rate of head development gives evidence for the existence of a physiological differential along the longitudinal axis of body at least in the anterior zooid region.

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[4] However, such difference in rate between 4/8-1 and 4/8-5 pieces as mentioned below is not unusual but is within the range of normal variations. In this species a difference as great as this has been frequently found in later experiments (unpublished data).

According to Sivičkis' classification of form of axial gradients in triclads (Sivičkis, 1931*a*), the present species seems to belong to his *P. velata* group rather than to his *P. dorotocephala* group, since the pieces taken from any level of this species produce 100 per cent of perfectly reconstituted individuals. But, as described above, the rate of eye formation of this species shows a distinct U-shaped double gradient with the lowest rate at or near the zone of fission. In Sivičkis' classification this U-shaped gradient is that which has been taken as a characteristic feature of the *dorotocephala* group. However, the only criterion used for his classification was the capacity for development of eyespots, no other characters in reconstitution being taken into account, though, as he insisted, the appearance of eyespots may be a convenient index, but not necessarily the best, for determination of the form of physiological axial gradients. In fact a gradient of capacity for head development is merely one of the various expressions of the axial differential in physiological organization of the planarian body. In addition to the data presented in this paper, the evidence from the growth rate of anterior and posterior new tissues and from differential susceptibility to lethal solutions as indicated by the disintegration processes in whole animals (Child, 1932) shows that the present material belongs to the *dorotocephala* group, although its head frequencies are extremely high and show no distinct axial differential as do those of other members of the group (cf. Child and Watanabe, 1935; Watanabe, 1935*b;* Sivičkis, 1923).

In normal worms the variations in steepness of the gradient of rate are usually limited within a certain range under average normal conditions of laboratory environment. However, as shown in Table 2, in a certain stock the rate was nearly equal at all levels of the body. Similar decrease in gradient has also been found in head frequency in *D. dorotocephala* in certain stocks the head frequency of which was extraordinarily high at all levels with very slight gradient (Watanabe, 1941). The worms of this stock were evidently not in normal condition. They did not live long under laboratory conditions, while worms of other stocks collected from the same locality lived indefinitely there and showed similar gradients of rate of eye formation to those in worms from other localities. Such anomalies probably result from certain conditions in the environment in nature prior to experimentation.

Abeloos and Buchanan are of opinion that the rate of head development has no relation to length of piece in *Planaria gonocephala* (Abeloos, 1930) and *Phagocata gracilis* (Buchanan, 1933). But the data presented by these authors do not appear to exclude the possibility of a relation between length of piece and rate of head development. As has been shown in Table 3, in the present species as in *D. dorotocephala* the rate is little or not at all affected by length of piece at the anterior level, but at more posterior levels of the anterior zooid it increases with increase in length of piece and attains a maximal value when the length of piece extends beyond 3/8 of the postcephalic length. These data indicate that, at the posterior levels of the anterior zooid, the differentiation processes in the formative tissue at or near the anterior cut surface are more or less retarded by a factor originating from the posterior section of the piece. The inhibitory action on head development apparently undergoes a decrement in effectiveness with increasing distance from the level of origin. This hypothesis is supported also by the results from posterior half-section (Tables 4 and 5). At the posterior level of the anterior zooid half-section made at a short distance posterior to the anterior cut retards head differentiation and growth of new anterior tissue on the side of the half-section, resulting in a temporary asymmetry in head form of the regenerates.

According to this view, the maximal rate of such long pieces as 3/8 and 4/8 of the postcephalic length indicates the rate of differentiation of the head-forming tissue itself at the level of the anterior cut without any effect of the inhibiting factor. Consequently, an axial differential of rate of eye formation in 4/8 pieces from five different levels (Table 6) indicates the existence of a gradient of head-differentiating activity along the main axis. This finding seems to be rather significant for the analysis of axial gradients of reconstitution in planarians.

It has been suggested in work with *D. dorotocephala* and *D. tigrina* that the head-frequency gradient is a result of axial differentials of the relative intensities of two antagonistic factors which are concerned in reconstitution of the planarian head: the one, a positive determining factor being the condition and activity of the cells which give rise to the head; the other, a negative inhibitory factor, the temporary physiological effect of posterior section (Child and Watanabe, 1935; Watanabe, 1935*b*). As regards head frequency, however, no decisive direct evidence has yet been given for an axial differential of either the head-determining factor or the head-inhibiting factor separately. When the inhibitory factor is eliminated by making the posterior cut at a distance sufficiently posterior to the anterior cut or by sufficient delay of posterior section, pieces of these species develop all normal heads, and no differential in head frequency is found. Moreover, data at hand do not permit us to determine whether the relation between head frequency and level of body is due to a decrease in the anterior direction of the effect of posterior section or to an increase in the anterior direction of ability to antagonize it, although the inhibitory factor is evidently effective over a greater length of piece in the posterior than in the anterior region of the anterior zooid. Now in the light of the evidence presented in this paper there can be no doubt as to the existence and form of the anteroposterior differential head-forming activity. This in turn suggests that it may be possible to obtain more definite information concerning the axial relation of the head-inhibiting factor. As regards this negative factor in this species, further data will be presented in a later paper.

The average standard deviations of mean times given in the preceding tables show, first, that variation in rate of head development increases from anterior to posterior levels of the anterior zooid (Tables 1 and 6) and, second, that at the same level it increases generally with decrease in length of piece (Table 3). These variations of mean times may be associated, at least in part, with the variations in physiological condition to which the pieces examined are subjected because of their position in the axial organization of the body and with the variations in excitation resulting from posterior section. Differences in complexity, presence of one or more than one zooid, and stage of physiological development of zooids are doubtless concerned in the variations and irregularities of mean times in the posterior zooid region.

SUMMARY

1. With a Japanese planarian known as *P. gonocephala* as material, mean times from section to appearance of eyespots were determined in pieces of equal length from different body-levels and in pieces of different lengths from the same levels. From these mean times values for relative rates of differentiation of new heads were obtained as percentages of a standard mean time taken as 100.

2. In pieces of equal length the rate of eye formation decreases from anterior to posterior levels of the anterior zooid and increases again in the posterior zooid region. This axial differential corresponds to differential susceptibility as indicated by disintegration in whole animals, but there is no corresponding head-frequency gradient; in this

species, under average normal conditions of laboratory environment, all pieces from all levels develop normal heads.

3. At the anterior level immediately posterior to the head, length of piece from 1/16 to 4/8 has little or no effect on rate of eye formation, while at more posterior levels the rate increases as length of piece increases from 1/16 to 3/8 and attains a maximal rate in 3/8 pieces for each level.

4. A transverse half-section at a very short distance posterior to the anterior cut end of the piece retards eye formation and the rate of growth of new tissue on the side of the half-section in pieces from the posterior region of the anterior zooid but is not effective in pieces from the anterior region just behind the head.

5. The maximal rate obtained in 4/8 pieces differs according to levels of body. This fact confirms, independently of the effect of length of piece, the evidence for the antero-posterior differential in rate of head development.

6. Variations in mean times of appearance of eyespots increase in general from anterior to posterior levels of the anterior zooid and with decrease in length of piece.

7. In 1/8 pieces from all levels of an aberrant stock, rate of eye formation is very high, variation small, and both nearly equal at all body-levels. In these respects the pieces from all levels of this aberrant stock resemble the pieces from the region immediately behind the head of ordinary stocks.

LITERATURE CITED

ABELOOS, M. 1927. La Vitesse de régénération de la tête chez *Planaria gonocephala* Dugés. Compt. rend. Acad. Sci. Paris, 184:345.

———. 1930. Recherches expérimentales sur la croissance et la régénération chez les planaires. Bull. biol. France et Belgique, 64:1.

BUCHANAN, J. W. 1933. Regeneration in *Phagocata gracilis* (Leidy). Physiol. Zoöl., 6:185.

CHILD, C. M. 1932. Experimental studies on a Japanese planarian. I. Fission and differential susceptibility. Sci. Repts. Tohoku Imp. Univ., Biol., 6:314.

CHILD, C. M., and WATANABE, Y. 1935. The head frequency gradient in *Euplanaria dorotocephala*. Physiol. Zoöl., 8:1.

FISHER, R. A., and YATES, F. 1938. Statistical tables, for biological, agricultural and medical research 8:90. London: Oliver & Boyd, Ltd.

HYMAN, L. H. 1931. Studies on the morphology, taxonomy and distribution of North American Triclads. IV. Trans. Amer. Micros. Soc., 50:336.

———. 1939. North American Triclad Turbellaria. IX. *Ibid.*, 58:264.

SIVIČKIS, P. B. 1923. Studies on the physiology of reconstitution in *Planaria lata*, with a description of the species. Biol. Bull., 44:113.

———. 1931*a*. A quantitative study of regeneration along the main axis of the triclad body. Arch. zool. ital., 16:430.

———. 1931*b*. A quantitative study of regeneration in *Dendrocoelum lacteum*. Arb. d. II Abt. d. ungar. biol. Forschungsinstitut, 4:1.

———. 1933. Studies on the physiology of regeneration in triclads. V. D. Univ. Mat.-Gamtos Fak. Darbai, 7:369.

———. 1934. The effect of the regeneration activity upon the rate of regeneration of eyes in *Planaria lugubris*. *Ibid.*, 9:13.

WATANABE, Y. 1935*a*. Rate of head development as indicated by time of appearance of eyes in the reconstitution of *Euplanaria dorotocephala*. Physiol. Zoöl., 8:41.

———. 1935*b*. Head frequency in *Euplanaria maculata* in relation to the nervous system. *Ibid.*, p. 371.

———. 1937. Susceptibility of planarian pieces to various concentrations of Murray-Ringer solution Sci. Repts. Tohoku Imp. Univ., Biol., 11:279.

———. 1941. Effects of modified Ringer solution on head reconstitution in *Euplanaria dorotocephala*. Physiol. Zoöl. 14:316.

DIFFERENTIAL MODIFICATIONS OF COELOM DEVELOPMENT IN THE STARFISH, PATIRIA MINIATA

(Thirty-two figures)

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IT IS well known that modifications of form and proportions result from exposure of eggs and embryos to toxic agents and conditions. A considerable body of evidence based on experiment indicates that modifications of early development produced by particular agents are very generally not specifically different from those produced by other agents. Within a certain range of concentration or intensity of action similar modifications are produced by many different agents and conditions which unquestionably act in different ways on living protoplasms. In general, these modifications are differential in character; that is, alteration of form and proportions is not localized in a particular region or organ primordium but is graded in relation to the axiate pattern of the organism or part concerned and its changes in the course of development. The differential character of the modifications results from a differential susceptibility generally characteristic of physiological axes; and this, in turn, is associated with graded, apparently quantitative, axial difference in physiological condition, involving metabolism and substrate. The most intensely active regions are most susceptible to the direct toxic action of a large number of agents and are consequently most inhibited in development by this action, and the degree of inhibition decreases with decreasing activity along the axis. With the lower ranges of toxic action of many agents secondary developmental modifications, opposite in direction as regards form and proportion, result from differential tolerance, differential acclimation or conditioning, and differential recovery after temporary exposure. These modifications usually follow some degree of primary differential inhibition. In general, the regions most susceptible to the more extreme toxic actions show, in the course of time, greater tolerance and greater or more rapid acclimation or conditioning with continuous exposure, and recover more rapidly or more completely after temporary exposure to the less extreme actions of agents. In early development of various organisms polar, ventrodorsal, or dorsiventral patterns of the whole organism and of organ systems all show differential modifications of similar character, apparently dependent on quantitative, rather than specific, differences in condition along the axes. Axiate pattern may be completely obliterated by differential inhibition, or the regional differences along an axis may be increased beyond the normal by the secondary modifications.

Early echinoderm development shows a wide range of differential modification with many agents, both chemical and physical, apparently because specific regional differentiations along the axes have not proceeded very far in the egg and embryo. At any rate, the modifications show little evidence of specific regional differences. Certain modifications have been regarded by some investigators as indicating such differences and as specific effects of particular agents, but this is perhaps still open to question.[1]

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[1] For experimental data on differential modification of echinoderm development by various agents and its physiological basis and for citations and discussion of literature see the following: Child, 1915; 1916*a;* 1916*b;* 1936*a;* 1936*b;* 1938; 1940; 1941, chap. vi.

The present paper is primarily concerned with differential modifications of coelom development, but many of the figures show differential modifications of larval form and proportions. The figures of experimentally modified development represent the most advanced stages attained by the individuals figured. It is a point of considerable interest that the greater the degree of differential inhibition the earlier the stage at which development stops. The figures are diagrammatic but are drawn from sketches of the living animals, immobilized when necessary, so that ocular micrometer measurements could be made as a basis for figures. The ciliated bands, so far as they are present, are indicated in broken lines. Parts of gut or coeloms seen through other parts of these organs are represented in dotted line. Experimental procedure in each case figured is given in the legend.

The data were obtained at the Hopkins Marine Station, Pacific Grove, California, in the winter of 1936; publication has been delayed by other work. The kindness of the director, Dr. W. K. Fisher, and of the staff in providing facilities and material is again gratefully acknowledged.

COELOM DEVELOPMENT IN *Patiria* UNDER NATURAL CONDITIONS

After invagination of entoderm is completed, the apical or anterior region of the archenteron enlarges greatly, its wall becomes thinner, and mesenchyme cells are given off from it. With these changes rate of dye reduction in low oxygen increases greatly in the enlarged region, the increase being greatest in the median anterior region (Fig. 1) and apparently somewhat greater anterodorsally than ventrally. This suggests that the enlargement and change in form of this region are associated with a considerable activation of its cells. Dye reduction becomes more rapid there than in any other part of the later gastrula except the extreme apical ectoderm in some individuals. With extension of this archenteric region ventrally to meet the stomodeal invagination the region of most rapid dye reduction appears to shift somewhat ventrally (Fig. 2). The two anterior coeloms develop as lateral budlike outgrowths extending more or less posteriorly from the enlarged archenteric region (Fig. 3). As they develop, rate of dye reduction becomes temporarily higher in them than in the median region (Fig. 4). The two coelomic buds separate completely from the entoderm, undergo elongation, and from one, somewhat more frequently the left, but often the right, the tubular madreporic canal extends dorsally to the ectoderm and opens to the exterior. The median region of the anterior archenteron becomes the foregut or esophagus (Fig. 5). During these changes a posterior coelomic bud develops from the midgut, more commonly on the left side (Fig. 5), and the anterior coelom of the same side extends posteriorly and unites with it (Figs. 6 and 7).[2]

DIFFERENTIAL MODIFICATION OF COELOM DEVELOPMENT BY LITHIUM CHLORIDE

The anterior coeloms.—The modifications of anterior coelom development show a wide range of variation in different individuals of a single lot, all in the same container and exposed to the same concentration of LiCl at the same stage and for the same time. This variation probably results in part from individual differences in susceptibility, but even without crowding there may be considerable local differences in environment in the same container. For individuals of nonmotile early stages, more or less aggregated in groups, environment as regards LiCl, oxygen, and CO_2 must become increasingly different from that of widely

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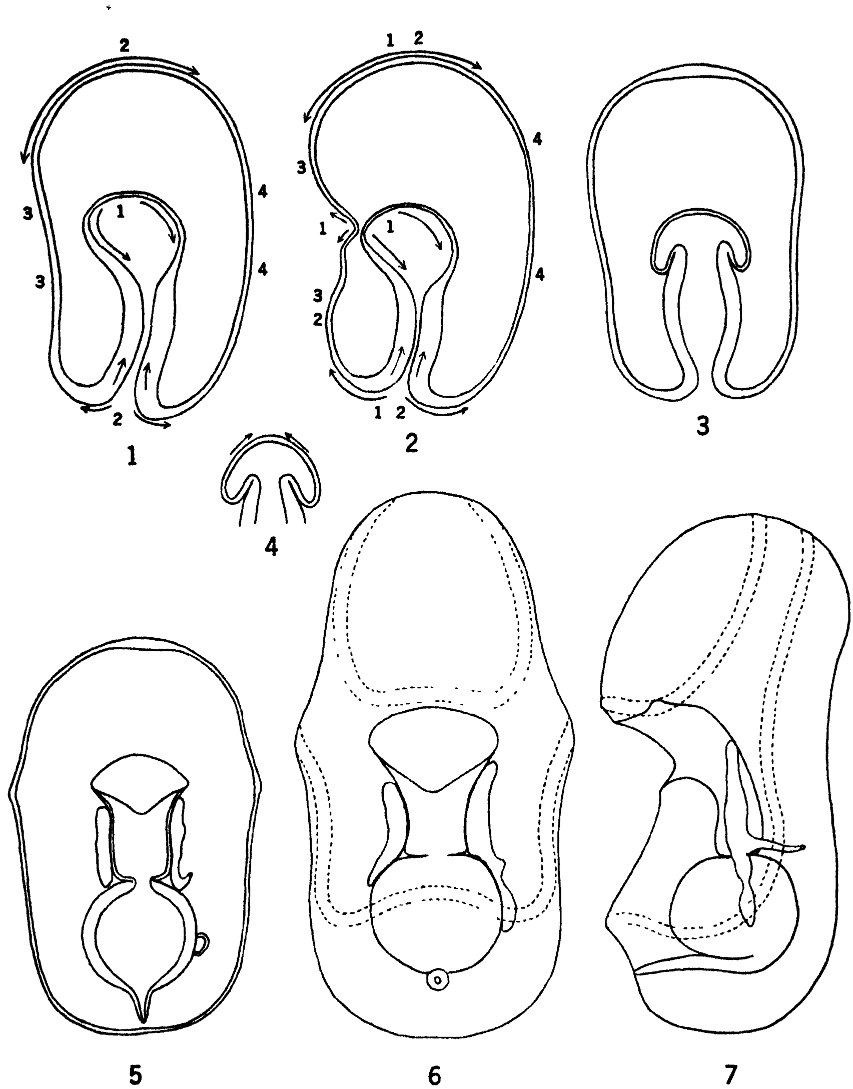
[2] For studies on asymmetry in *Patiria* see Newman, 1921*a*, 1921*b*, 1922, 1925.

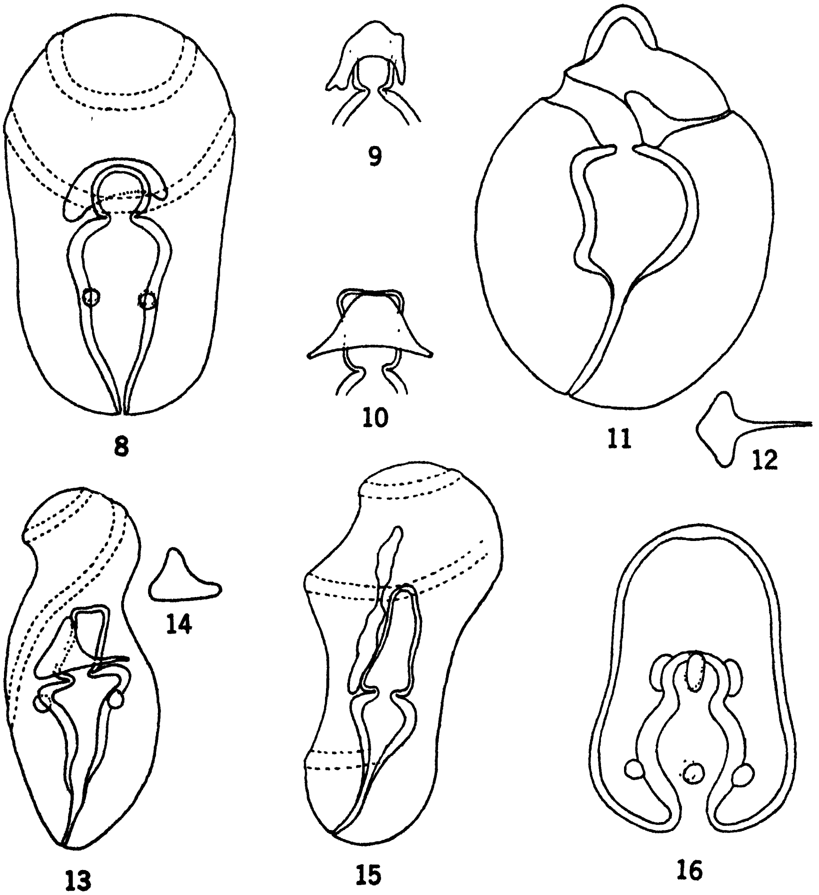


Figs. 1–7.—Normal development. Figs. 1 and 2, differential dye reduction in gastrula stages; arrows indicate directions of progress of reduction from regions of most rapid reduction, numerals, relative times of reduction in different regions, two numerals in the same region indicating variation in time in different individuals (from Child, 1936*a*). Fig. 3, formation of anterior coeloms. Fig. 4, course of dye reduction at stage of coelom buds. Fig. 5, later stage in ventral view, showing anterior coeloms, the left one with madreporic canal, and the posterior coelom of the left side of midgut. Figs. 6 and 7, ventral and lateral outlines of advanced larvae.

scattered individuals. Aggregations of swimming stages may also alter their environment. Slight difference in physiological condition permitting individuals to swim at the water surface instead of moving about in contact with the bottom may determine considerable differences in developmental modification. Dye reduction indicates that oxygen content is normally low in the blastocoel, and with slight differences in environment it may be above or below a critical level. Renewal of solution or change of water may alter the environment of many individuals in the lot. Consequently it is not certain that the modifications in LiCl solutions are all due entirely to LiCl, and the same is true for other agents; but comparison with controls makes it sufficiently evident that the agent is the primary factor in bringing about the modifications. They are all effects—first of differential inhibition, with perhaps secondary modifications. In the lithium modifications described below the secondary modifications represent differential recovery after return to water.

The usual modification of anterior coelom development with moderate inhibition is approach of the loci of coelom development to the median plane anterodorsally on the foregut, with resulting formation of a single sac with the usual asymmetry more or less evident or reversed or with complete bilateral symmetry. With more extreme inhibition a single rounded median sac may develop at the anterior end of the foregut. From the differentially inhibited coelom a madreporic canal may or may not develop, and, if it develops, may end blindly without reaching the ectoderm; a bilaterally symmetrical single coelom often gives rise to two canals, left and right, bilaterally localized. In Figure 8, viewed ventrally, the right side of the single coelom is the larger and there is no canal; in Figure 9, a dorsal view, the left side is larger, the short blind outgrowth from it is probably an inhibited madreporic canal; the foregut is distinctly inhibited. Figure 10, a dorsal view, shows a case of bilaterality with two canals, both reaching the surface. The single coelom of Figure 11 is anterodorsal and median, somewhat bilateral in form when viewed anteriorly (Fig. 12), and the single madreporic canal is median. These modifications represent primarily results of differential inhibition of the foregut region. In the individuals of Figures 8–10 the foregut ends blindly without extending ventrally, and no stomodeum develops. The inhibition is greatest in the median anterodorsal region of the foregut, corresponding closely to the region of most rapid dye reduction in the later gastrula. The animals figured were all returned to water before gastrulation, and coelom development took place only after more or less recovery from lithium effects. Recovery, however, was not complete, and the gradient level from which coelom develops is nearer the median plane anterodorsally on each side, so that a single coelom with more or less indication of its double character, instead of two separate sacs, develops. Normally the anterodorsal side of the foregut undergoes much greater increase in size than the other side as the extension ventrally takes place (Fig. 7). Since the ventral extension does not take place in the cases of Figures 8–10, inhibition of the foregut is evidently greater anterodorsally than ventrally, and approximation and union of the regions of coelom development usually result. Even in cases like Figure 11, in which the foregut extends ventrally and connects with a small mouth, its anterodorsal side is more inhibited than the ventral, as comparison with the normal control (Fig. 7) shows.

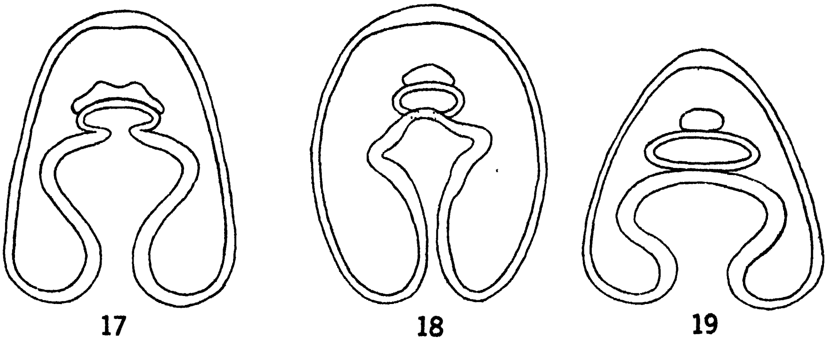
A single anterodorsal coelom is not the only modification resulting from differential inhibition by lithium. Not infrequently a single asymmetrical or symmetrical coelom develops on the ventral side of the foregut. In the individual of Figures 13 and 14 the single ventral coelom is asymmetrical with a madreporic canal extending around the left side of the foregut and opening laterodorsally on the left side. Here the dorsiventrality of the foregut is apparently reversed; and it extends more or less dorsally in the blastocoel, that is, there is little or no recovery dorsally and the coelom develops from the less susceptible ventral side. Another case of single ventral coelom, median and without canal, resulting from longer exposure to a somewhat lower concentration of LiCl, is shown in Figure 15. Here the differential inhibition was sufficient to reverse dorsiventrality of the foregut and localize coelom develop-



Figs. 8–16.—Modifications resulting from exposure to LiCl for certain periods in early stages, followed by return to water. Fig. 8, LiCl, m/25, 11 hours from 32 to 64 cells, 3 days water. Fig. 9, dorsal view of asymmetrical coelom and inhibited foregut, probably an inhibited canal on left; same treatment as in Fig. 8. Fig. 10, dorsal view of bilateral coelom and inhibited foregut; same treatment as Fig. 8. Fig. 11, lateral view of differentially inhibited larva with median dorsal coelom and median canal; LiCl m/30, 21½ hours from 2-cell stage, 7 days water. Fig. 12, coelom and canal of Fig. 11 in anterior view. Fig. 13, ventral anterior coelom with canal on left, foregut much inhibited with ventral side longer than dorsal, 2 posterior coeloms; LiCl m/25, 11 hours from 32 to 64 cells, 8 days water. Fig. 14, coelom of Fig. 13 in ventral view. Fig. 15, ventral coelom with more advanced development, no posterior coelom, 3 ciliated bands, posterior band only on ventral side; LiCl m/30, 38½ hours from 64 cells, 10 days water. Fig. 16, larva completely radial in form with 4 anterior and 4 posterior coeloms; LiCl m/25, 19½ hours from 32 to 64 cells, 4 days water.

ment ventrally, but after return to water the coelom itself underwent considerable recovery. In general, ventral coelom development is more frequent with high concentrations or long exposures, but a few individuals with ventral coeloms may appear in the same lots with those developing single anterodorsal coeloms.

Occasionally three or four coeloms develop, apparently equally spaced about the foregut (Fig. 16). In these cases dorsiventrality is apparently completely obliterated in the foregut by differential inhibition, and ventrodorsality is more or less completely obliterated in the whole larva. In the larva of Figure 16 foregut and all other parts of the larval body are completely radial in form. In some of these cases, however, slight ventrodorsality of form in the ectoderm or the beginning of stomodeal invagination indicates that the coeloms are dorsal, lateral, and ventral; but in forms like Figure 16 there is no evidence of ventrodorsality. A few individuals with a single dorsal and a single ventral coelom have been seen, and two individuals with two coeloms oblique to the median plane and opposite or nearly opposite. In these the foregut shows no evidence of dorsiventrality, but the larval form is slightly bilateral. These forms with three or four coeloms about a radial foregut evidently represent more or less recovery after complete or almost complete obliteration of dorsiventrality in the foregut, but how localization about the polar axis and number of coeloms are determined is not evident.



FIGS. 17–19.—Modifications resulting from continuous exposure to LiCl m/40 from 24-hour blastula; 8 days LiCl; coelom completely anterior to greatly inhibited foregut and no posterior coelom.

In all cases described thus far except that of Figure 15 the material was returned to sea water from LiCl before gastrulation began. At the time of return to water development was retarded, and the blastulae were somewhat smaller than the controls. With the higher concentrations and longer exposures there was more or less exogastrulation after return to water. Entogastrulation after lithium treatment resulted in forms smaller than controls with ectoderm more inhibited than entoderm, which consequently appeared relatively "too large." Enlargement of the anterior region of the archenteron was usually more or less inhibited and smaller than normal, and, as the figures show, the foregut usually remained blind with little or no ventral extension or even some extension toward the dorsal side (Figs. 13 and 15), and stomodeal development was completely inhibited; the more inhibited individuals often remained completely radial (Fig. 16).

With continuous exposure to concentrations of LiCl, not lethal before development stops but producing distinct differential inhibition, forms like Figures 17–19 result. Morphological ectodermal development does not proceed beyond the earlier gastrula stages, and ectodermal inhibition decreases from the apical region basipetally. The entoderm is relatively "too large" but usually elongates less, and the anterior enlargement is smaller than in controls and at best does not develop beyond the stage of a rounded or ovoid sac, often apparently no longer open into the midgut (Figs. 18 and 19). The anterior coelom is usually single without any trace of asymmetry and almost or entirely anterior to the foregut. It may be partly double, as in Figure 17, or merely slightly elongated laterally (Figs. 18 and 19) and does not develop further. Coelom development in these cases may represent secondary modification involving some degree of differential tolerance or conditioning to LiCl.

With more extreme inhibitions the anterior coelom may lose epithelial character at an early stage and dissociate into free cells like mesenchyme, or the whole anterior archenteron may undergo more or less complete dissociation before coelom development; more or less dissociation of entodermal cells into the blastocoel may occur during, or even before, gastrulation, the latter case resembling immigration of mesenchyme in echinoids (Child, 1940).

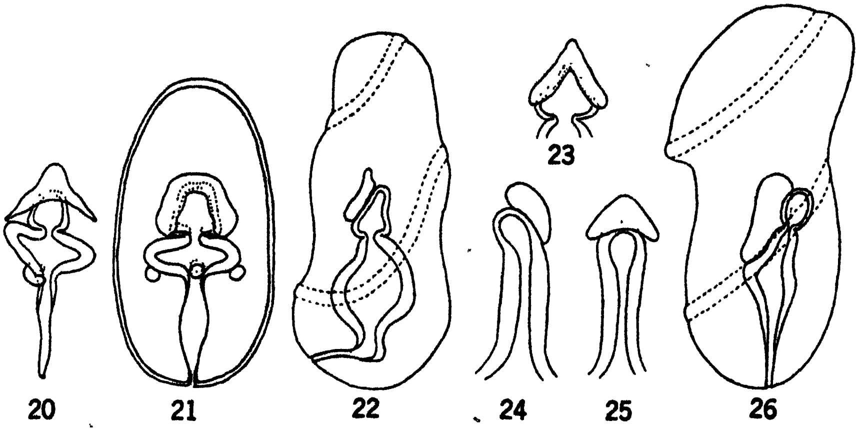
The posterior coelom.—The usual effect on posterior coelom development of moderate inhibition by temporary exposure to LiCl is development of two, three, or more posterior coelomic buds at the same level about the midgut (Figs. 8, 13, and 16). In one individual six of these buds were present, equally spaced. With increase in number of posterior coeloms evidence of asymmetry is usually absent, even though it may be present in anterior coelomic development (Fig. 8). The modifications suggest that some degree of dominance of one side, usually the left, is present in the uninhibited animal and that under inhibiting conditions this dominance is obliterated. Longer exposures to concentrations which still permit anterior coelomic development after return to water usually inhibit posterior coelom development completely (Figs. 11 and 15), that is, the anterior entodermal region recovers more completely than the midgut. With continuous exposure to inhibiting concentrations permitting some degree of anterior coelomic development there is usually no posterior coelomic development (Figs. 17–19), the midgut apparently never attaining the stage at which this development becomes possible.

DIFFERENTIAL MODIFICATION OF COELOM DEVELOPMENT BY OTHER AGENTS

In the course of experiments on differential modification of development by other agents it was found that the same series of modifications of coelom development appear as with LiCl.

Nile blue sulphate.—In order to determine whether or to what extent Nile blue sulphate might be toxic and produce differential inhibition of echinoderm development, it was used in very low concentrations, which stained the embryos and larvae deeply. One part in ten thousand in sea water did not dissolve completely or precipitated in part. Various dilutions of the part in solution were used; but, within a wide range, difference in concentration makes no great difference, for the dye is accumulated in the cells even from highly dilute solutions. In the higher concentrations it becomes toxic somewhat earlier because it accumulates more rapidly. Coelomic modifications are like those produced by LiCl. Union anterodorsally of the two anterior coeloms with more or less complete disappearance of asymmetry and failure of the foregut to extend ventrally are the most frequent. With more extreme effects a single entirely anterior coelom or a ventral coelom develops. Two, three, or four posterior coelomic buds appear frequently. Figures 20–23 show a few characteristic modifications. In all these cases the foregut is evidently inhibited, does not extend ventrally, and stomodeum is absent. In Figure 20 the single anterior coelom is bifurcated with two symmetrical canals and the posterior coelom is elongated, extending about halfway around the gut, probably in consequence of continuity of two bud loci. In Figure 21 the posterior portions of the anterior coelom extend ventrally almost around the inhibited foregut, but canals are entirely absent; four posterior coelom buds are present. Except for the bilaterality of the anterior coelom and perhaps the foregut, the larva is radial in form. The single anterior coelom of Figure 22 is ventral (Fig. 22), but bilateral (Fig. 23) and posterior coelom development is completely inhibited. These three individuals are from the same lot; for other gradations of modification the figures for LiCl will serve as well as any.

Neutral red.—Lots kept for 2 days in very low concentrations of neutral red with gradual further dilution by precipitation show the same series of coelomic modifications: single asymmetrical or bilateral anterior coeloms (Figs. 24, 25) and some cases of ventral coelom (Fig. 26). The entoderm in these forms is often much inhibited, as in the figures, probably because it stains deeply; anterior coelom development appears to be a secondary modification of the anterior entodermal region following return to water. The general form of the larva often shows distinct evidence of secondary modification, the preoral region becoming much enlarged and the anterior coelom relatively very large, as compared with the gut. The animals apparently are able gradually to dispose of some of the dye in some way if not too much injured at first, and this process is also differential. Posterior coeloms have not been seen in any of the neutral red modifications; the more posterior regions of the entoderm are apparently not able to dispose of the dye as rapidly as the anterior region.

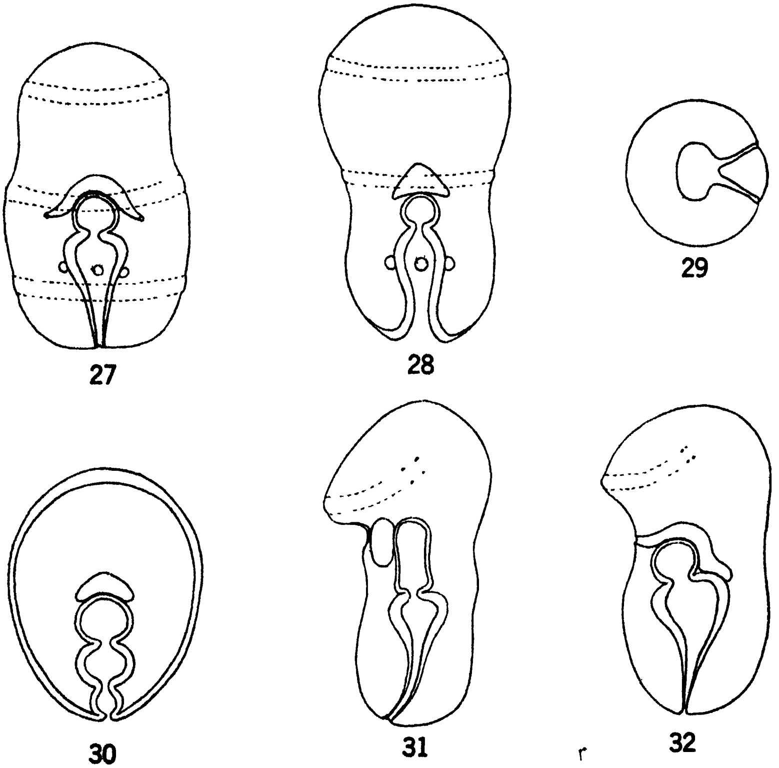


FIGS. 20–26.—Modifications by Nile blue sulphate and neutral red. Figs. 20–23, Nile blue sulphate, saturated (?) sea-water solution diluted 1/2, 19 hours from 32 to 64 cells, 5 days water. Fig. 20, dorsal outline of bilaterally symmetrical anterior coelom and gut, foregut greatly inhibited, blind, without ventral extension, no stomodeum, posterior coelom partly surrounding midgut; Fig. 21, dorsal view, anterior coelom bilateral, partly surrounding inhibited, almost radial foregut, no stomodeum, 4 posterior coeloms; Fig. 22, larva with ventral coelom; Fig. 23, coelom and foregut of Fig. 22 in ventral view. Figs. 24–26, 2 days weak neutral red from 19-hour blastula, 4 days water. Figs. 24 and 25, anterodorsal coeloms with inhibited gut; Fig. 26, a ventral coelom somewhat on the right side.

Acidified sea water.—Acidification to pH 6.6 by HCl also modifies coelom development in the same ways as LiCl. The effective agent in this case may be CO_2 rather than hydrogen ion.

Hypotonic sea water.—Eighty per cent sea water gives a high frequency of coelomic modifications, primarily differential inhibitions with more or less secondary modification in some cases and indistinguishable from the LiCl modifications. In 90 per cent sea water the modifications are, in general, less extreme and less frequent.

Tobacco smoke.—This was found to be extremely effective in producing differential inhibition. A dry finger bowl covered immediately and left standing for a day after smoke is puffed into it makes sea water differentially inhibitory. Even finger bowls kept after washing in a room in which there was considerable smoking produced differential inhibition of coelom development and of larval form. Coelomic modifications show the same series as with LiCl and other agents. Figures 27–32 are examples. The larva of Figure 27 is completely radial in form with three ciliated bands extending horizontally around the body, a common result of differential inhibition. The foregut is merely a rounded sac, but the anterior coelom is bilateral with two canals, the only evidence of bilaterality in the larva. Four posterior coeloms are present. In the animal of Figures 28 and 29, also radial in form, the anterior coelom is



Figs. 27–32.—Smoke. Figs. 27 and 28, bilateral anterior coeloms, 4 posterior coeloms; Fig. 29, anterior view of coelom of Fig. 28; Fig. 30, coelom entirely anterior to inhibited foregut, larva entirely radial except for slight bilaterality of coelom, no posterior coelom; Fig. 31, median ventral coelom between stomodeal invagination and inhibited foregut, no posterior coelom; Fig. 32, coelom median, anterodorsal, opening with ventral canal and pore. These animals were in smoked finger bowls during early development with one or more changes of water shortly before or in early gastrulation.

entirely anterior to the small inhibited foregut, and two canals are present symmetrically localized near the median plane (Fig. 29). This individual also has four posterior coeloms. Figure 30 shows a more extreme inhibition with slight secondary modification of the anterior region of the archenteron following changes of water after the inhibition by products of smoke. In Figure 31 the coelom is ventral, lying between the foregut and a slight stomodeal invagination. The single median anterior coelom of Figure 32 opens in the median ventral region, and the stomodeum is completely inhibited. Ventral pores have been seen occasionally with other agents but never frequently under any conditions used thus far.

Old stock.—Animals kept 9 days in a laboratory aquarium before use were very slow in discharging eggs and sperm when removed from water, and development was differentially inhibited, with the same series of coelomic modifications as with other inhibiting conditions.

Complete inhibition of coelom development.—Development may be stopped at practically any stage according to degree and rapidity of inhibiting action by any of the agents mentioned above and by various others. Entodermal modifications with complete inhibition of coelom development constitute a further graded series with increasing degree of inhibiting action. More or less enlargement of the anterior archenteron may occur but never reach the stage of coelom development, and the entoderm remains without evidence of bilaterality. The enlargement may be completely inhibited, the wall of the anterior region remaining thick like the rest of the entoderm, and there is no visible difference or boundary between foregut and midgut. More or less dissociation of the anterior region of the archenteron may occur after or during invagination, or cells may even dissociate from the entodermal region before invagination begins.

DISCUSSION

The inhibiting action on the entoderm of all agents and conditions used is very distinctly differential, the anterior region of the archenteron, which normally undergoes enlargement and apparently marked activation, being most inhibited. Moreover, even in this region the inhibition is differential, the anterodorsal region being most inhibited. The differential modifications of coelom development are physiologically essentially similar to the differential modifications of the planarian and vertebrate head and the sea-urchin pluteus. In the planarian head under inhibiting conditions all degrees of approximation to the median plane of eyespots and of cephalic lobes and corresponding obliteration of median regions of the cephalic ganglia appear, according to degree of inhibiting action.[3] With differential inhibition of the head in fishes and amphibia there is a similar progressive approximation and union in the median plane of loci of development of bilateral cephalic organs as inhibiting action increases.[4] In the differentially inhibited sea-urchin larva the anal arms are approximated to the median plane, a single median arm may develop, or arm development may be completely inhibited and bilaterality obliterated.[5] Evidently, all these modifications result from differential susceptibility to inhibiting agents and conditions, depending on differences in physiological condition which are graded rather than sharply defined or localized. Since these differences in susceptibility in the planarian and vertebrate head, the sea-urchin larva, and the starfish archenteron are not specifically different for different agents, the conclusion appears justified that they are associated with, and depend on, quantitative differences in physiological condition rather than on qualitative differences in constitution, even though such differences may be present in greater or less degree. If they are present, they are apparently not the factors concerned in these differential modifications.

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[3] Child, 1921; Child and McKie, 1911; Child and Watanabe, 1935.

[4] See, e.g., Stockard, 1907, 1909; Bellamy, 1919; Child, 1941, chap. vii.

[5] Child, 1916*b*, 1938, 1940*a*, 1941.

Development of a single coelom on the ventral side of the foregut apparently results from inhibition of lateral, dorsal, and anterior regions to such a degree that they cannot give rise to coeloms. After return to water or after repeated change of water in the case of smoke, dorsal and anterior regions do not recover sufficiently before development stops to develop a coelom, but the less susceptible ventral side is sometimes not too much inhibited to develop a coelom. Development of three or four coeloms about the foregut probably results from obliteration of dorsiventrality; there is no evidence of dorsiventral difference in such cases.

From the modifications it is evident that anterior coelom development can take place from lateral, dorsal, anterior, or ventral regions of the foregut and that the number of coeloms may range from one, without or with evidence of bilaterality or asymmetry, to four. Evidently the locus of coelom development is not a fixed, specifically determined region but depends on the gradient in physiological condition in the anterior part of the archenteron and foregut and on the alterations of this gradient by external factors. The median anterior and anterodorsal region of the anterior archenteron represent the high region of the gradient system, and coelom development is localized in a region on each side defined by the gradient system. Under inhibiting conditions the gradient level for coelom development is shifted nearer the median plane anterodorsally or anteriorly or, with certain degrees of inhibition, to the ventral side; and with obliteration of dorsiventrality the apicobasal gradient in the anterior archenteron is the only localizing factor remaining, and several coeloms may develop at the same level about the foregut.

The asymmetrical position of the posterior coelom, usually on the left, and the appearance of two, three, four, and in one case six coelom buds about the midgut, suggests that one side is usually dominant under natural conditions and that with obliteration of asymmetry this dominance is abolished. That one side develops more rapidly and inhibits the other was suggested by Newman (1925). The frequent development of equal paired madreporic canals from differentially inhibited anterior coeloms may result from a similar elimination of dominance of one side over the other.

In view of the hypothesis that lithium has a specific action in enhancing, extending, or in some way making more effective a vegetal concentration gradient of substance (Runnström), it is a point of particular interest that the modifications of development produced by LiCl do not differ in any characteristic manner, so far as could be determined from hundreds of preparations, from those produced by other agents. They do not provide any evidence for specific action of lithium on morphological entodermal or coelomic development. At the time of its enlargement and apparent activation the anterior archenteric region is differentially inhibited in the same way as by other agents, and the same series of developmental modifications results. In short, whatever the constitution of this region, the morphological modifications of development by LiCl and other agents thus far used apparently depend on quantitative physiological differentials rather than on localizations of specific substances or metabolisms.

SUMMARY

1. With LiCl and various other agents which inhibit development similar series of modification of localization and development of the coeloms appear in the starfish, *Patiria miniata*.

2. The modifications of anterior coelom development consist in approximation to the median plane anterodorsally of the loci of coelom origin with more or less complete union of

right and left coelom, decrease or obliteration of asymmetry, a single bilateral coelom with two madreporic canals or with none; with certain degrees of inhibition followed by some recovery, a single coelom ventral to the foregut; with obliteration of dorsiventrality in the foregut followed by recovery, development of three or four coelom buds about the foregut; with continuous exposure to certain degrees of inhibition, completely radial larval form and a single coelom entirely anterior to the greatly inhibited, completely radial foregut. With still more extreme inhibition anterior coelom development is completely inhibited, and dissociation of cells from the apical archenteron or from the whole entodermal region may take place.

3. Modifications of posterior coelom development by differential inhibition consist in obliteration of the normal asymmetry; and with recovery after return to water two, three, four, in one case six, posterior coelom buds develop at the same level about the midgut. With more extreme inhibition posterior coelom development is completely inhibited. The midgut region is less capable of recovery after return to water than the foregut.

4. The modifications result from graded differential inhibitions and differential recoveries, not from specific local actions of agents. Since they are not specific for LiCl or any other agent used, they are believed to depend on quantitative differentials in physiological condition, and presence of such differentials is indicated by other methods.

LITERATURE CITED

Bellamy, A. W. 1919. Differential susceptibility as a basis for modification and control of development in the frog. I. Biol. Bull., 37:312.

Child, C. M. 1915. Axial gradients in the early development of the starfish. Amer. Jour. Physiol., 37:203.

———. 1916*a*. Axial susceptibility gradients in the early development of the sea urchin. Biol. Bull., 30:391.

———. 1916*b*. Experimental control and modification of larval development in the sea urchin in relation to the axial gradients. Jour. Morph., 28:65.

———. 1921. Studies on the dynamics of morphogenesis. XI. Jour. Exper. Zoöl., 33:409.

———. 1936*a*. Differential reduction of vital dyes in the early development of echinoderms. Arch. f. Entw'mech., 135:426.

———. 1936*b*. A contribution to the physiology of exogastrulation in echinoderms. *Ibid.*, p. 457.

———. 1938. Certain differential modifications of asteroid and echinoid development. Annot. Zool. Japon., 17:205.

———. 1940. Lithium and echinoderm exogastrulation, etc. Physiol. Zoöl., 13:4.

———. 1941. Patterns and problems of development. Chicago: University of Chicago Press.

Child, C. M., and McKie, E. V. M. 1911. The central nervous system in teratophthalmic and teratomorphic forms of *Planaria dorotocephala*. Biol. Bull., 22:39.

Child, C. M., and Watanabe, Y. 1935. The head frequency gradient in *Euplanaria dorotocephala*. Physiol. Zoöl., 8:1.

Newman, H. H. 1921*a*. On the occurrence of paired madreporic pores and pore canals in the advanced bipinnaria larva of *Asterina* (*Patiria*) *miniata*, etc. Biol. Bull., 40:118.

———. 1921*b*. The experimental production of twins and double monsters in the larvae of the starfish *Patiria miniata*, etc. Jour. Exper. Zoöl., 33:321.

———. 1922. Normal versus subnormal development in *Patiria miniata*. Biol. Bull., 43:1.

———. 1925. An experimental analysis of asymmetry in the starfish, *Patiria miniata*. *Ibid.*, 49:111.

Stockard, C. R. 1907. The artificial production of a single median cyclopian eye in the fish embryo by means of sea water solutions of magnesium chloride. Arch. f. Entw'mech., 23:249.

———. 1909. The development of artificially produced cyclopian fish. "The magnesium embryo." Jour. Exper. Zoöl., 6:285.

THE ALTERATION OF DEVELOPMENTAL PATTERN IN THE SAND DOLLAR BY PILOCARPINE[1]

(Forty-three figures)

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OTHER work on the dynamics of echinoderm development (Child, 1936*a*, 1936*b*, 1940; Rulon, 1938, 1941) has shown that such agents as lithium and sodium thiocyanate affect early developmental pattern primarily through inhibitory action. The inhibition of certain active regions may be followed secondarily by recovery effects, depending upon the experiment, to produce modifications of widely different types. Differential conditioning may also take place in low concentrations. By recognizing differential inhibition, recovery, and conditioning in the modified embryo it is possible to tell something of the time of origin, the localization of particular fields, the development of symmetry, and other factors operating in the progressive development of the normal pattern.

With data on the effects of inhibitory action available, it seemed desirable to test the effect on developmental patterns of an agent which reputedly has a stimulating action. Since there were many indications that pilocarpine might fill this need, a number of experiments were performed with this substance as the test agent.

MATERIAL AND METHODS

The eggs used in these experiments were taken from the ripe females of the sand dollar, *Dendraster excentricus*, collected near Pacific Grove, California, during the height of the breeding season. Fertilization was accomplished by adding sperm suspension and was usually 95–100 per cent effective. The solutions of pilocarpine hydrochloride were made up directly in sea water. Approximately 200 eggs were exposed to 150 cc. of solution. All cultures were kept in covered finger bowls on a water table and examined at regular intervals. Parallel control experiments were always run in normal sea water.

NORMAL DEVELOPMENT

For comparative purposes a few of the more important stages or landmarks in the normal development are here described. Fuller accounts may be found elsewhere (Child, 1940; Rulon, 1941).

Cleavage is total and by 7–8 hours after fertilization at the temperatures used in these experiments reaches the middle blastula stage (Fig. 1). Here the larvae have fairly thick walls, little apicobasal differentiation, and are beginning to show ciliary movement. By 12 hours after fertilization the larvae are top-swimming, the walls, with the exception of the vegetal region, are much thinner, mesenchyme has appeared in the basal region, and there may be indications of gastrulation (Fig. 2). At 24 hours the larvae are definitely bilateral with a flattened ventral and a rounded dorsal ectoderm (Fig. 3). The archenteron has grown considerably by this time, and its apical end is directed toward the future stomodeal region. The earliest indications of anal arms may be found at the 24-hour stage where they appear as slight lateral protuberances on the posterior or anal regions of the ventral surface. The oral lobe is also turning ventrally at this stage. At 48 hours the larva has attained the middle pluteus stage with long anal arms, a definite skeleton, stomodeum, flattened oral lobe, and short oral arms (Fig. 4, lateral; Fig. 5, anal). At 60 hours the oral lobe is narrower and the arms longer (Fig. 6). Development usually stops between 60 and 70 hours after fertilization, and the larvae begin to die.

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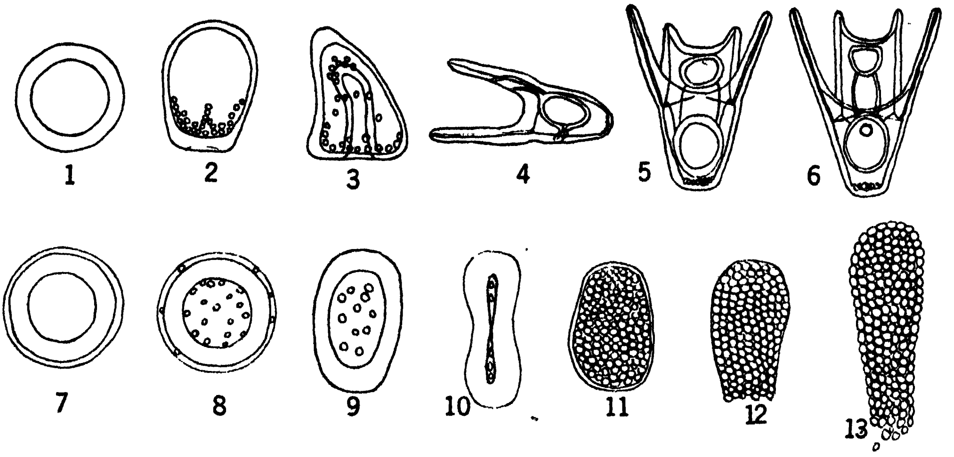
[1] The writer wishes to express his gratitude to Dr. W. K. Fisher, director of the Hopkins Marine Station, Pacific Grove, California, for the use of the laboratory space and facilities which made this work possible.



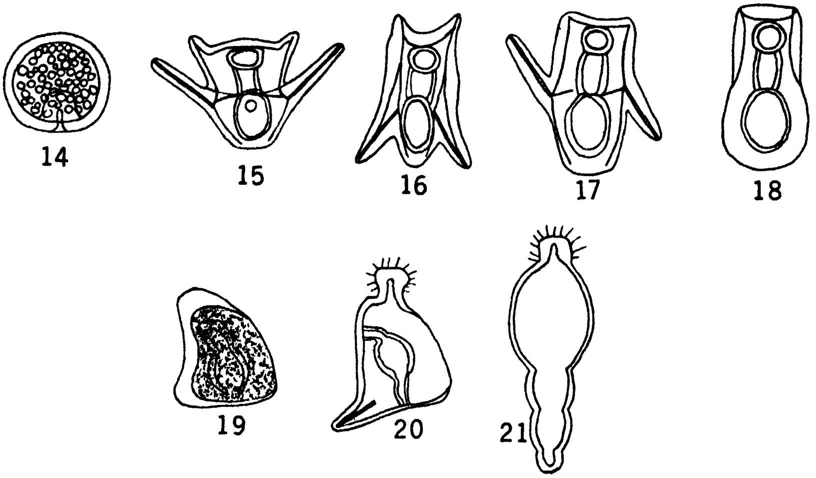
FIGS. 1–13.—Figs. 1–6, normal development of the sand-dollar larva from 7 to 60 hours after fertilization at a temperature of approximately 15° C. Figs. 7–13, inhibited larvae after 12–48 hours in M/100 pilocarpine.

EXPERIMENTAL

1. *The modification of pattern with high concentrations applied immediately after fertilization.*—When fertilized eggs were placed directly in M/100 pilocarpine and the developing larvae examined at 12-hour intervals, a clear picture of the effects of continuous exposure to this concentration was given. At the end of the first 12 hours the larvae appeared as slow-moving, thick-walled blastulae still inside their fertilization membranes (Fig. 7). The cells of these forms seemed larger than normal and showed little or none of the apicobasal differentiation that appeared in the normal blastula of this age (Fig. 2). In many, cells were seen escaping into the blastocoel from the whole of the internal lining, indicating an anaxiate condition (Fig. 8). After 24 hours in this solution the larvae were usually elongate, thick-walled forms, considerably flattened and partly or entirely filled with large cells (Figs. 9–10). A number were giving off cells to the outside from the vegetal region, while in a few there were indications of gastrulation. All were actively moving, although usually near the bottom of the culture dishes. At 36 hours they appeared chiefly as thin-walled bags of cells (Fig. 11) or as bags with cells escaping from the vegetal end (Fig. 12). At 48 hours the larvae were, for the most part, curious, elon-

gate bottom forms, filled with large cells and with cells protruding from the vegetal region (Fig. 13). They were often quite active and gave the appearance of wriggling about. At 72 hours most of the larvae had disintegrated by a progressive dissociation into separate cells from the vegetal region.

When the larvae were returned to normal sea water after the first 12 hours in M/100 pilocarpine, the developmental history was very different. After 12 hours in sea water these larvae were usually free-swimming, early gastrulae with an excess of internal cells (Fig. 14). At 60 hours a number appeared almost normal, but in many the oral and anal arms were at widened angles (Fig. 15). In others the anal arms were at aberrant angles, askew, or absent (Figs. 16–18). Some extremely inhibited forms, ranging from cell-filled blastulae to inhibited gastrulae (Fig. 19), were found near the bottom of the culture. In many of the inhibited forms a ciliated knob appeared at the apical region (Fig. 20). This knob—a common feature of apical differential recovery—was a rudimentary oral lobe. A few exogastrulae appeared with this treatment, and some of these had also developed a ciliated knob (Fig. 21).

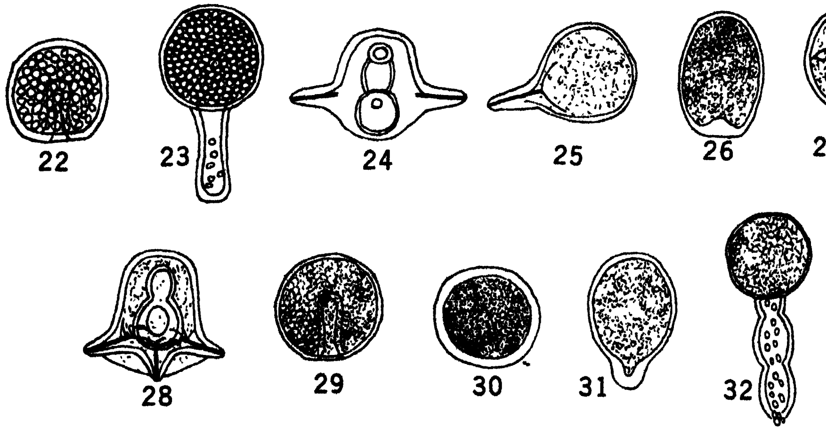


FIGS. 14–21.—Larvae developing in normal sea water after an initial exposure of 12 hours to M/100 pilocarpine.

Treatment for 24 hours with M/100 pilocarpine followed by return to normal sea water for the remainder of the larval period yielded forms which showed considerably more inhibition. After 24 hours in normal sea water following treatment most of the larvae appeared as free-swimming, radially symmetrical forms, filled with large cells, and showing all degrees of gastrulation and exogastrulation (Figs. 22–23). Twenty-four hours later they had reached their maximum development. There were no normal plutei, and bilaterality was evident in only a few. Many were little more than bags of cells, but a number showed a bit of skeleton and short anal arms (Fig. 24). None showed differentiation of the oral lobe, but a number possessed a gut although filled with cells and cellular debris. In some the anal arms were at aberrant angles or askew, while in others only a single arm developed (Fig. 25). A number of the larvae were completely radial (Figs. 26–28), and, in some, short anal arms developed around the entire circumference (Fig. 28).

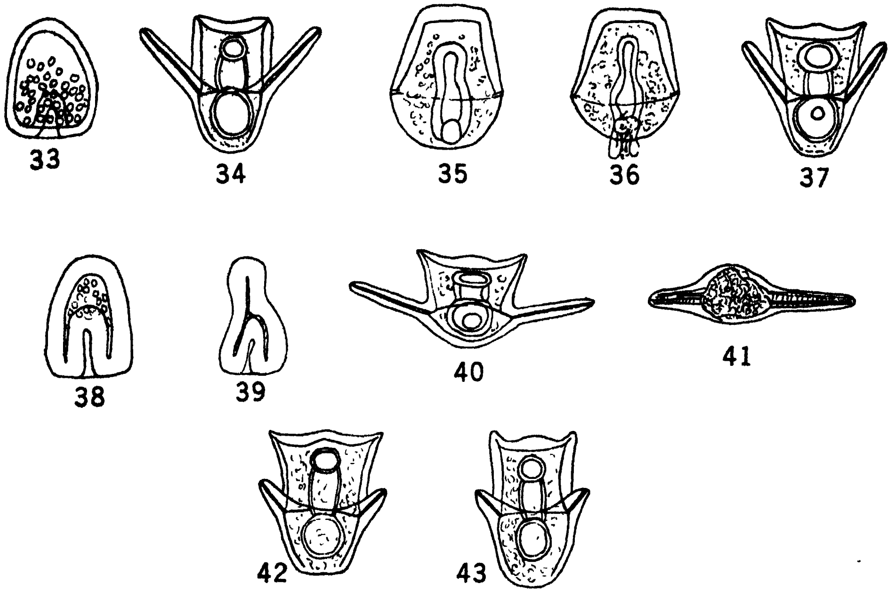
Treatment for the first 48 hours usually produced larvae which failed to develop either bilaterality or skeleton when returned to sea water. All were filled with cells or cellular debris, although many showed some invagination (Fig. 29). Some developed no further than blastulae (Fig. 30), while others showed an evagination, underdeveloped entoderm (Fig. 31). In a number an evaginated gut was distinctly overdeveloped (Fig. 32).

2. *The modification of pattern with medium concentrations applied immediately after fertilization.*—When fertilized eggs were exposed continuously to M/500 pilocarpine, the effects were less drastic than in the higher concentration. In the early stages this concentration had a stimulating effect on rate of cleavage, but by 11–12 hours the test blastulae were definitely inhibited in that the walls were thicker and there was less ciliary action than in the controls. An excess of mesenchyme was also found in these forms. At 24 hours in M/500 pilocarpine the larvae were chiefly top-swimming, early gastrulae (Fig. 33), which were less advanced than the controls and had noticeably thicker walls. Occasionally there was a form which had failed to gastrulate, and near the bottom of the cultures were found a number of cell-filled blastulae. After 48 hours in the test solution most of the larvae appeared as thin-walled bags of cells (Fig. 11), although in some there was a slight indication of gastrulation. It was noted in many cases that the invaginated gut became dissociated into separate cells with continued exposure to this agent. Bilaterality was also inhibited. After 72 hours most of the larvae were dead, and those that remained alive were little more than radial or irregular bags of cells. Some still had a trace of undissociated gut. Development in this solution proceeded no further, and death usually resulted by 96 hours.



FIGS. 22–32.—Larvae developing in normal sea water after an initial exposure of 24–48 hours to M/100 pilocarpine.

When the larvae were returned to normal sea water after the first 11–12 hours in M/500 pilocarpine, the later development was not very different from that of the controls. After 60 hours in sea water most of the larvae approached normal plutei. In a number, however, the anal arms were askew, asymmetrical, or at widened angles. A number showed an excess of mesenchyme. In some the oral lobe was inhibited, while in others the oral and anal arms were both inhibited. When the larvae were returned to sea water after the first 24 hours the effects of the agent were more noticeable. After 48 hours in sea water these forms, in general, approached the pluteus stage but presented many evidences of inhibition and recovery. In most, the oral lobes had differentiated but little, and the anal arms were at widened angles (Fig. 34). In some the anal arms were asymmetrical or askew. Others were extremely inhibited, and all showed an excess of mesenchyme. A number showed the ciliated apical knob found in treatment with higher concentrations (Fig. 20). Forty-eight hours' exposure followed by return to sea water resulted in larvae which had undergone little differentiation. Most of the forms remained almost or quite spherical and so heavily filled with cells and internal debris that it was next to impossible to see any internal structures. In some, however, there was a trace of bilaterality, and in a few a bit of gut was seen.



Figs. 33–43.—Fig. 33, larva after first 24 hours in M/500 pilocarpine. Fig. 34, larva developing in normal sea water after 24 hours' initial exposure to M/100 pilocarpine. Figs. 35–36, larvae developing in M/1,000 pilocarpine. Fig. 37, larva developing in M/2,500 pilocarpine. Figs. 38–39, larvae resulting from 12½ hours' exposure to M/100 pilocarpine beginning at the middle blastula stage. Figs. 40–41, larvae resulting from 12½ hours' exposure to M/100 beginning at the middle blastula stage and a return to sea water for 48 hours. Figs. 42–43, larvae resulting from continuous exposure to M/1,000 pilocarpine beginning at the late blastula stage.

3. *The modification of pattern with low concentrations applied immediately after fertilization.*—Continuous exposure to M/1,000 pilocarpine resulted chiefly in swollen forms with an excess of internal cells. These larvae had definite bilaterality but no anal or oral arms. Occasionally a trace of skeleton was seen in the anal-arm region (Fig. 35). A number

showed a portion of the gut evaginated from the anal region (Fig. 36). Although extensive differentiation was lacking, the oral lobe was larger than in the controls, and the animal as a whole presented a more flattened appearance. Continuous exposure to M/2,500 pilocarpine gave chiefly swollen forms with a widened oral lobe. Excess mesenchyme was present, and there was usually no development of oral arms. The anal arms were shorter than in the control and often were at widened angles (Fig. 37). Many normal plutei developed under continuous exposure to M/5,000 pilocarpine. In a number, however, the oral lobe was broader than normal. Practically all had well-developed oral arms which often extended at an angle from the oral lobe. Most of the larvae developing in this concentration were top-swimming as compared to the bottom forms of higher concentrations. In general, when larvae were removed from these low concentrations of pilocarpine to sea water, development proceeded toward the normal pluteus. In many cases, however, the larvae showed the effect of the agent in a widened oral lobe and shortened oral and anal arms.

4. *The modification of pattern with various concentrations applied at later stages in the developmental history.*—After 12 hours in M/100 pilocarpine, larvae which had been placed in this agent in the middle blastula stage (Fig. 1) appeared chiefly as large, active, thick-walled, cell-filled, flattened gastrulae (Figs. 38, 39). When returned to normal sea water they developed after 48 hours into forms showing various degrees of inhibition and recovery. In most of them, anal arms were present, although they ranged from extremely long structures to mere stumps. These arms were commonly at widened angles, askew, or duplicated. In most of them the oral lobe was not developed, but in some it had undergone considerable development and increase in width (Fig. 40). Occasionally a ciliated apical knob appeared. Most forms had an excess of mesenchyme, and a number had excess skeleton (Fig. 41), although in some the skeleton was diminished or lacking. Most showed evidence of bilaterality, but others seemed to be entirely radial.

Continuous exposure to M/1,000 pilocarpine beginning with the 12-hour form (Fig. 2) permitted considerable development by 70 hours (Figs. 42, 43). Almost all had definite bilaterality. An excess of internal cells was evident, and the larvae were somewhat flattened and swollen. The majority had short anal arms and an enlarged oral lobe which often had definite oral arms. The skeleton was more or less developed, and gut was usually present. When returned to sea water after 40 hours' exposure to the pilocarpine, the larvae developed very much as with continuous exposure except that the oral and anal arms became slightly longer.

DISCUSSION

It cannot be emphasized too strongly that these, or any, experiments on the modification of development must have as their aim an attempt at a better understanding of the normal pattern. The dynamics of development are extremely obscure, and anything but the broadest assumptions must necessarily at this time be tentative. However, it seems evident that the developmental modifications representing differential inhibition, differential conditioning, and differential recovery are primarily expressions of dynamic factors of developmental pattern with graded axial differentials, undergoing definite progressive change in the course of development, but with little or no evidence of regional specificity (Child, 1940, and earlier papers).

In general the effects of pilocarpine parallel the effects of other inhibitory agents, although there is a slight acceleration of cleavage in low concentrations. It is not to be expected that all inhibitory agents will have an exactly similar effect. Some agents may exert a rapid action and rapidly be broken down or inactivated by the living protoplasm. Others act more slowly. Some (e.g., KCN) may exert their inhibitory action through the inactivation of respiratory enzymes, while others act in other ways. It may be that no two agents have exactly the same effect, but in general it is found that, regardless of the nature of the agent, the more active (physiologically) regions are more inhibited than the less active in high concentrations, become conditioned to inhibitory agents more rapidly in low concentrations, and recover (unless badly injured) more quickly and completely when the agent is removed.

The first noticeable effects of pilocarpine on the developing larva are the thickened wall of the blastula and an excessive number of internal cells (Figs. 8, 9, 14, 22). In low concentrations these internal cells are seen to come from the vegetal region of the blastocoel lining, and the increased number may be related to a greater susceptibility of this region at this particular stage of development. Child (1936*a*) showed with the aid of vital dyes a region of rapid reduction at this site in the normal larva at the time of formation of primary mesenchyme and entoderm. With high concentrations of pilocarpine internal cells become dissociated from the whole of the lining of the blastocoel and with continuous exposure the entire larva usually assumes the appearance of a cell-filled bag with free cells escaping externally from the vegetal region (Fig. 13). Such forms certainly approach an anaxiate condition, although at least partial return of the primary axis may take place on return to sea water (Figs. 31, 32). The cause of dissociation of cells externally from the vegetal region is not entirely clear. It may be related to an activation of that region or to a surface effect of pilocarpine which produces greater dissociation basally because the cells are slower there in attaining complete epithelial form.

With continuous exposure following fertilization the internal cells form little or no skeleton in concentrations of from M/100 to M/1,000. If returned to sea water after an exposure of 12–36 hours following fertilization, there may be sufficient recovery to form some skeleton. Often the skeletal pattern is aberrant and incomplete (Figs. 16, 17, 24, 25, 28). This may be because of a more or less complete obliteration of orienting factors in the ectoderm, which is much inhibited by these concentrations, and the pattern partly or wholly destroyed through direct inhibition or altered by irregular recovery. Anomalous distribution of mesenchymal elements, related to contact with substratum or nearness of other larvae, may also be a factor in the distortion of pattern. An excess of skeleton may develop, however, if 12-hour larvae are exposed for a short time to pilocarpine (Fig. 41). The increase in skeletal elements in this case may be the result of differential recovery of the activated mesenchyme following return to sea water.

These experiments have shown ventrodorsality to be relatively stable. In general, it develops and persists in M/1,000 pilocarpine—a concentration which, on continuous exposure, prevents or markedly inhibits the development of skeleton or oral and anal arms (Figs. 35, 36). Ventrodorsality can, however, be obliterated with continuous or short-time exposure to higher concentrations (Figs. 11, 12, 13, 26, 27, 28, 29, 30, 31, 32). Short-time exposures, immediately after fertilization, may prevent the development of ventrodorsality, yet permit the development of skeleton and anal arms (Fig. 28). Child (1936*a*) showed with vital dyes that the ventral side is the more active in a ventrodorsal gradient, and in his experiments with lithium (1940) he noted that ventrodorsality may be completely obliterated if the larvae are treated in the early stages. The higher concentrations of pilocarpine give essentially similar results. With somewhat less extreme differential inhibition the ventrodorsality becomes evident, but the ventral side remains flat or may become convex instead of becoming deeply concave as in normal development and the arm angle is wide, even approaching 180° (Figs. 15, 24, 34, 35, 36, 37, 40, 42, 43). Some of these forms probably represent a degree of differential recovery of ventral side and mesenchyme (Figs. 15, 24, 34, 40), while others represent a degree of differential conditioning (Figs. 35, 36, 37, 42, 43).

The polar gradient is extremely stable. Concentrations of pilocarpine which inhibit the development of ventrodorsality and prevent all differentiation (except cilia formation, etc.) do not, in general, completely destroy the apicobasal gradient pattern. This is shown by eggs which have been in M/100 for 48 hours or more. On return to sea water the basal region often differentiates into entoderm, which may become much enlarged through recovery (Fig. 32). Such exogastrulae, however, fail to show any differentiation of the ectoderm. Evidences of the original polar-activity gradient are also shown in the development of the ciliated knob through differential recovery of the apical end of larvae returned to sea water following exposure to high concentrations. Such a knob (rudimentary oral lobe) indicates a region of high activity near the apical end which can recover and develop when the inhibitory conditions are removed.

In general the apical region or oral lobe is highly susceptible in the early larva and is easily inhibited by various agents including pilocarpine. The appearance of the ciliated knob in larvae treated for a short time with high concentrations and returned to sea water indicates a high capacity for recovery of this region. The higher activity of this region may also be demonstrated through differential conditioning. On continuous exposure to low concentrations larvae may develop exceedingly large oral lobes, although the solution is strong enough to inhibit oral and anal arms as well as to prevent the development of a concave ventral surface (Figs. 35, 36, 37, 42, 43).

The reaction of entoderm to pilocarpine is quite similar in many ways to that found with such agents as lithium and thiocyanate. Continuous exposure to high concentrations prevents the differentiation of entoderm or may cause the cells of the presumptive entoderm to become dissociated. In lower concentrations (M/500) invagination of entoderm takes place, but the cells of the invaginated gut may become dissociated with continuous exposure. Still lower concentrations permit gut to form and invagination to take place. A 12-hour exposure to high concentrations following fertilization seems to have a greater effect on ectoderm than on entoderm. A high percentage of exogastrulae is produced, however, by 24 hours' exposure (Fig. 23). These exogastrulae differ from those produced by some other inhibitory agents in that the ectoderm ordinarily undergoes little recovery. Long-time (48-hour) exposure to high concentrations followed by return to sea water produces a number of exogastrulae with a definitely enlarged gut and a diminished ectoderm (Fig. 32). Such entodermized forms, when produced by lithium, have been interpreted by Child to mean that the basal regions of prospective ectoderm have been inhibited to the physiological level of entoderm of early stages and in consequence of decreased apical dominance undergo the activation characteristic of entoderm, as gastrulation approaches.

SUMMARY

1. Continuous exposure of fertilized sand-dollar eggs to M/100 pilocarpine retards cleavage and permits the development of only radial elongate bags of cells showing ciliary movement and a progressive dissociation into separate cells from the vegetal region.

2. Exposure of fertilized eggs for 12 hours to M/100 pilocarpine, followed by return to sea water, permits the development of plutei with anal arms at widened angles, asymmetrical, askew, or absent. Many distinctly inhibited, cell-filled larvae also appear with this treatment. Among the most interesting of these are some with a ciliated apical knob (rudimentary oral lobe), which represents a degree of differential recovery.

3. Exposures to M/100 pilocarpine for 24–48 hours, followed by return to sea water, tend to prevent the development of bilaterality, to inhibit gastrulation, and to produce a large number of cell-filled exogastrulae.

4. A solution of M/500 pilocarpine has a slight stimulating action on early cleavage, but after 11–12 hours' exposure distinctly inhibitory effects are noted. Larvae gastrulate in this concentration, but continued exposure produces a dissociation into separate cells of the invaginated entoderm. Development of ventrodorsality, oral lobe, and anal arms is also inhibited by continuous exposure. Removal of the developing larvae to sea water at certain intervals results in forms showing varying degrees of differential inhibition and recovery.

5. Continuous exposure to M/1,000 results chiefly in swollen, bilateral forms with an excess of internal cells, no anal or oral arms, and only a trace of skeleton. Continuous exposure to M/2,500 gives chiefly swollen, bilateral forms with widened oral lobes and shortened anal arms. The most noticeable effect of M/5,000 is an enlarged oral lobe. The enlarged oral lobes probably indicate differential conditioning.

6. Exposure of middle blastulae for 12½ hours to M/100 pilocarpine followed by return to normal sea water results in many aberrant larvae. The oral lobe is usually not developed, although in some cases it is considerably widened. Anal arms show many aberrancies. Most larvae have an excess of internal cells and many have excess skeleton. Most are bilateral, but some have retained their earlier radial symmetry.

7. Continuous exposure to M/1,000, beginning with the late blastula stage, results in larvae which usually show bilaterality, an excess of internal cells, a swollen appearance, and an enlarged oral lobe.

8. The recognition of differential inhibition, recovery, and conditioning resulting from pilocarpine treatment has aided in the discernment of certain critical periods and controlling factors in following the progressive development of a dynamic pattern in the larval sand dollar.

LITERATURE CITED

Child, C. M. 1936*a*. Differential reduction of vital dyes in the early development of echinoderms. Roux Arch. f. Entw'mech., 135:426.

———. 1936*b*. A contribution to the physiology of exogastrulation in echinoderms. *Ibid.*, 135:457.

———. 1940. Lithium and echinoderm exogastrulation: With a review of the physiological-gradient concept. Physiol. Zoöl., 13:4.

Rulon, O. 1938. Modification of development in the sand dollar with NaCNS and Ca-free sea water. Anat. Rec., Suppl., 72, No. 4.

———. 1941. Modification of development in the sand dollar by NaCNS and Ca-free sea water. Physiol. Zoöl., 14:305.

BUFFERING CAPACITY AND COMPOSITION OF THE BLOOD SERUM AND REGURGITATED DIGESTIVE JUICES OF THE MORMON CRICKET (ANABRUS SIMPLEX HALD.)[1]

(Two figures)

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A SEARCH for more effective means of controlling outbreaks of the Mormon cricket, *Anabrus simplex* Hald., has disclosed, among other things, a lack of fundamental knowledge regarding the physiology of these insects. Recent studies have shown that the composition of insect body fluids may play an important role in the toxicity of insecticides. The investigation described in this paper was initiated to study the properties of the blood and regurgitated digestive juice of the Mormon cricket.

MATERIAL AND METHODS

The blood used in this study was obtained from adult crickets by puncturing the dorsal intersegmental membrane and collecting the exuded drops in a capillary tube. From 0.05 to 0.10 ml. of blood was obtained from each cricket, and approximately 6 ml., thus collected, was immediately centrifuged to remove the blood clots. Five-ml. portions of this serum were used for the determination of pH and buffering capacity, and in addition approximately 25 ml. of whole blood was collected for chemical analysis.

No special precautions were taken to prevent the possible loss of CO_2 from the sample to be measured, since Wigglesworth (1934, 1939) points out that there seems to be no chemical provision for its transportation in the insect blood and that CO_2 probably is eliminated from the insect body solely by the process of diffusion.

Bishop's data (1923) show that relatively large changes in the CO_2 tension of the honeybee body fluid are necessary to alter appreciably the pH values. It was found during this investigation that the pH of Mormon cricket blood remained constant for as long as 5 hours when held to 3°–4° C. after withdrawal, thus indicating that there was little change in the CO_2 equilibrium. When pH determinations were made on the blood of *Orthoptera* other than the Mormon cricket with methods described in this paper, values practically identical with those given by Bodine (1926) and others, for the same species, were obtained.

The crickets were starved for 24 hours to insure the absence of food material in the regurgitated digestive juice. Since they readily regurgitate their crop contents when handled, the juice was collected by holding a small vial under the mouth parts. From 0.05 to 0.10 ml. was obtained from each individual. For the determination of pH and buffering capacity approximately 6 ml. of juice was collected and centrifuged in order to remove any solids which might be present. Thirty ml. was collected for chemical analysis, this sample likewise being centrifuged to remove the solids present which interfered with the withdrawal of uniform aliquots. After centrifuging, both the blood and the regurgitated digestive juices were kept at 2° C. until analyses were completed.

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[1] Contribution from the Montana State College, Agricultural Experiment Station, Paper No. 127 ("Journal Series").

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Preliminary trials of several methods for measuring pH and obtaining titration curves resulted in the selection of the glass electrode. The Cameron pH meter was used to obtain all the pH determinations reported in this paper.

The relatively high concentration of protein and the presence of appreciable color in the two fluids ruled out the colorimetric procedure as an accurate method. The main difficulty encountered with the bubbling hydrogen electrode was the production of excessive foam, necessitating the frequent addition of a foam-breaker. The quinhydrone electrode is unsatisfactory for measuring pH values above 7.5 but proved useful, as did the hydrogen electrode, for checking the normal pH value of the two fluids against the glass electrode.

Most of the methods employed in the chemical analyses of the two fluids were based on the procedures used in the clinical analysis of mammalian blood, as described by Peters and Van Slyke (1932). Since the digestive juice contained small amounts of silica, the reducing agent of Fiske and Subbarow (1925) was used in the colorimetric determination of phosphorus. Under the conditions employed, this reducing agent—1, 2, 4-aminonaphtholsulfonic acid—does not produce a blue color with silicomolybdic acid.

EXPERIMENTAL DATA

CHEMICAL ANALYSIS

The chemical analyses of the regurgitated digestive juice and the blood serum are shown in Table 1.

The color of the regurgitated digestive juice when first withdrawn from the cricket varied from dark brown to black, and after exposure to the air it turned a uniform black. It contained 19 per cent total solids. Proteins and nonprotein nitrogenous compounds were present in approximately equal amounts and together make up over half the total solids. The relatively high percentage of nonprotein nitrogen may have resulted from the 24-hour starvation period to which the crickets were subjected. Phosphorus was present chiefly in the inorganic form, although organic phosphorus accounted for 6.3 per cent of the total. Hoskins and Harrison (1934) in a qualitative examination of the ventricular contents of the honeybee failed to detect any organic phosphorus. Expressed in milligram equivalents, the potassium content of the juice was approximately twenty-five times the sodium content, while in the blood the sodium content exceeded that of the potassium.

Analyses were made on the blood serum rather than on the whole blood, for clotting took place soon after the blood was drawn. The addition of materials commonly employed to prevent clotting in mammalian blood had no effect on that of the cricket. The amount of clotted material formed in the cricket blood was very much smaller than that formed in mammalian blood. Only 1.8 per cent of the total nitrogen in the blood was removed with the clot. Immediately after withdrawal the blood was a clear straw color, but on exposure to the air it gradually changed to a dark brown. According to Wigglesworth (1939), this darkening is due to the formation of melanin.

Wigglesworth (1934) has pointed out that insect blood is characterized by high concentrations of amino acids and phosphates, and the data presented in this paper show that this is true in the case of cricket blood. This fluid had about the same concentration of amino acids and a much higher concentration of phosphate than Bishop *et al.* (1925)

found in the blood of honeybee larvae. The amount of total nitrogen in cricket blood was approximately the same as in honeybee larvae, but the proportion of nonprotein nitrogen, other than amino nitrogen, was much higher in the cricket blood. Magnesium and calcium in the cricket blood were much less abundant than in that of bee larvae. From this comparison it is apparent that not only are there large differences between mammalian and insect blood but also between the blood of different insects.

TABLE 1

CHEMICAL COMPOSITION OF THE BLOOD SERUM AND REGURGITATED DIGESTIVE JUICE OF THE MORMON CRICKET, *Anabrus simplex* HALD.

	MILLIEQUIVALENTS PER LITER	
	Blood Serum	Digestive Juice
Na	21.9	3.0
K	15.3	74.6
Ca	3.0	38.4
Mg	1.4	23.2
PO_4 (total)	97.4	104.0
PO_4 (inorganic)	58.9	97.4
N (total)	930.0	1279.0
N (protein)	495.0	606.0
N (nonprotein)	434.0	672.0
N (amino)	187.0	517.0
N (nonprotein other than amino)	247.0	155.0
Cl	...	91.0
H* (calculated)	25.9	64.2
Total cations	67.5	203.4
Total anions	58.9	188.4
Total solids (gm. per liter)	94.8	197.5
Ash (gm. per liter)	5.68	11.0

* The figures given for H represent the H combined with the phosphate salts present in the two fluids at their normal pH values.

In both the digestive juice and the blood serum the total anions expressed as milliequivalents per liter are less than the total cations determined. However, chlorides, which were not determined in the blood, would add to the sum of the anions. It is probable that the two totals would not be equal in any case, for undoubtedly some of the cations were associated with organic anions such as amino acids and complex phosphorus compounds which were not determined. In calculating the total cations, account was taken of the hydrogen ions combined with phosphates at the normal pH values of two fluids.

pH VALUES AND BUFFER INDICES

The pH of the regurgitated digestive juice was 5.17. Hoskins and Harrison (1934) report pH values ranging from 4.6 to 6.3 for various parts of the digestive tract of the honeybee. Wigglesworth (1934) states that the gut contents of insects are slightly acid and that in general they tend to be more alkaline in phytophagous, and more acid in carnivorous, insects. The cricket is usually a phytophagous insect, but under some conditions it may become strongly carnivorous. The 24-hour starvation period prior to collecting the juice may have contributed to its strongly acid character.

The titration curve for the regurgitated digestive juice is shown in Figure 1. The points on the curve represent averages of five determinations. The greatest variation in individual values for any point on the curve was 0.20 pH. The values for most of the points varied less than 0.10 pH.

The buffering-capacity curve shown in Figure 2 was obtained by plotting pH against β values.[4] The values of β were determined by Van Slyke's (1922) procedure of drawing tangents to the titration curve. Small deviations from a smooth titration curve can easily result in large variations in β values when the tangent method is used. For this reason maximum points on the buffering-capacity curve should be viewed with skepticism, especially where biological systems are being investigated. In such systems conclusions drawn from pH measurements should be based on several replications, as exact measurements are difficult to obtain.

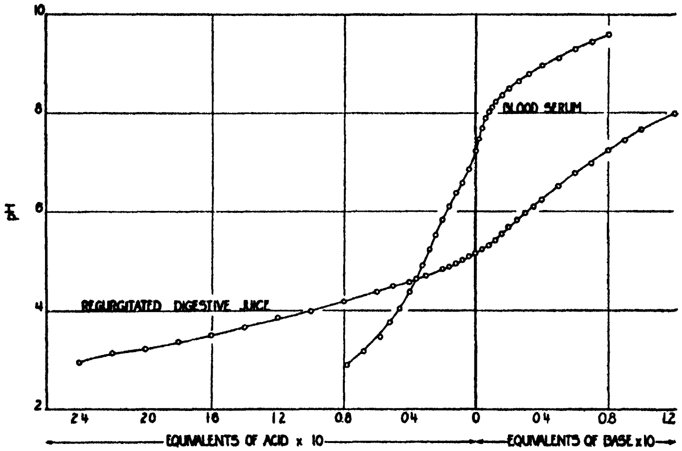

FIG. 1.—Titration curves for blood serum and regurgitated digestive juices of the Mormon cricket (*Anabrus simplex* Hald.).

No distinct inflection point occurred at pH 5.17—the normal pH of the digestive juice—or at any other point throughout the range investigated. At a pH of 5.17 the ratio of dibasic to monobasic phosphate is given by the equation

$$5.17 = PK' + \log \frac{B_2HPO_4}{BH_2PO_4}.$$

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[4] β being defined as follows:

$$\beta = \frac{dB}{dpH} \quad \text{or} \quad \frac{dA}{dpH},$$

where B and A are equivalents of base and acid per liter, respectively.

Taking the value of PK' as 6.9 (Cohn, 1927) and solving for the ratio B_2HPO_4/BH_2PO_4, it is found that at a pH of 5.17, over 95 per cent of the phosphate is present as the monobasic salt. Under these conditions the phosphate system has practically no buffering action, and one would not expect it to produce any maximum β value at this point. On the basis of the data available it is impossible to designate any individual buffering system, but this does not imply that such systems do not occur.

Wigglesworth (1934) states that the blood reaction in insects is usually very slightly acid and also that it may vary with sex and age. The pH of the Mormon cricket blood serum was found to be 7.23, which is somewhat higher than Bodine (1926) found for a number of other *Orthoptera*. The highest value that he reported for any insect investigated was 7.04 for the blood of the last instar larvae of *Pieris rapae*.

In the titration curve for the blood, shown in Figure 1, the points plotted are averages for five determinations. The variations in individual values were considerably less than those recorded for the digestive juice.

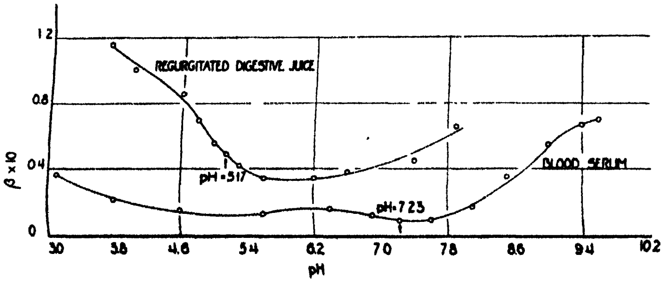

FIG. 2.—Buffering-capacity curves for the blood serum and regurgitated digestive juices of the Mormon cricket (*Anabrus simplex* Hald.).

It will be noted that smooth titration curves containing no clear-cut inflection points were obtained for both the blood and the digestive juice. No doubt this is due to the presence of a great many buffering systems and the consequent overlapping of their buffering effects.

Both fluids contain a high percentage of amino acids. The buffering effects of these amino acids would be spread over a wide range due to their varying PKa values. In this respect insect blood is not comparable to mammalian blood which has a low amino acid concentration.

The curve for the buffering capacity of the blood (Fig. 2) was obtained in the same manner as that for the digestive juice. It will be noted that the blood has little buffering capacity at its normal pH (7. 23).

There is a slight increase in the β values on the acid side of 7.23. These values become slightly lower in the neighborhood of pH 5 and then rise slowly. On the alkaline side the β values increase more rapidly. In other words, the blood has a greater buffering effect for bases than for acids. It may readily be shown that the greater buffering effect on the alkaline side cannot be due to the phosphate system. At a pH of 7.23 the ratio of dibasic to monobasic phosphate is 2.14 to 1, or 68 per cent of the phosphate is present as the dibasic salt. As the pH is increased above 7.23, the proportion of dibasic salt will become greater. At the same time the buffering capacity of the phosphate system will decrease and reach a minimum at a pH of approximately 9.5. The uniformity of the β values for the blood in the vicinity of its normal pH indicates that no single buffering system is responsible for the buffering capacity. When the buffering effects overlap to give uniform β values, it becomes difficult to differentiate between inflections on the titration curve due to a single buffer system and inflections due to slight errors of measurement. For example, in the case under discussion shifting the titration curve only 0.10 pH in the vicinity of 6.9 could produce a maximum in the buffering-capacity curve corresponding with the effect expected from a phosphate system. Consequently, it is only safe to conclude that the buffering capacity at the normal pH of the blood is probably due to a combination of buffering systems.

SUMMARY

A study of the regurgitated digestive juice and the blood serum of the Mormon cricket, *A. simplex* Hald., revealed the following facts:

1. The regurgitated digestive juice contained high concentrations of amino acids, phosphates, and potassium and a very low sodium concentration.
2. A buffering-capacity curve derived from a titration curve indicated that the juice did not show a maximum buffering effect at its normal pH of 5.17.
3. Sufficient data were not available to designate a buffering system at this point, but it was shown that the phosphate system could not exert much effect.
4. The blood serum likewise contained a high concentration of amino acids and phosphates. Expressed in milliequivalents the ratio of sodium to potassium was 1.4:1 compared with a ratio of 1:25 in the regurgitated digestive juice. The total and amino nitrogen content of the cricket blood serum was similar to that reported for the blood of honeybee larvae, but the inorganic constituents differed considerably.
5. The buffering-capacity curve showed that at the normal pH, 7.23, the blood had little buffering effect for acids and only slightly more for bases. Amino acids, which are not important buffering agents in mammalian blood, are probably responsible for a large part of the buffering action in cricket blood, due to their high concentrations.

LITERATURE CITED

Bishop, G. H. 1923. Body fluid of the honeybee larvae. Jour. Biol Chem., **58**:543–565.

Bishop, G. H., Briggs, A. P., and Ronzoni, E. 1925. Body fluids of the honeybee larvae. Jour. Biol. Chem., **66**:77–88.

Bodine, J. H. 1926. Hydrogen ion concentration in the blood of certain insects (*Orthoptera*). Biol. Bull., **51**:363–369.

Cohn, E. J. 1927. The activity coefficient of the ions in certain phosphate solutions Jour. Amer. Chem. Soc., **49**:173.

Fiske, G. H., and Subbarow, Y. 1925. The colorimetric determination of phosphorus. Jour.Biol.Chem., **66**:375–400.

Hoskins, W. M., and Harrison, A. S. 1934. The buffering power of the contents of the honeybee and its effect upon the toxicity of arsenic. Jour. Econ. Ent., **27**:924–942.

Peters, J. P., and Van Slyke, D. D. 1932. Quantitative clinical chemistry, Vol. 2. Baltimore: Williams & Wilkins Co.

Van Slyke, D. D. 1922. On the measurement of buffer values and on the relationship of buffer value to the dissociation constant of the buffer and the concentration and reaction of the buffer solution. Jour. Biol. Chem., **52**:525–570.

Wigglesworth, V. B. 1934. Insect physiology. London: Methuen & Co., Ltd.

———. 1939. The principles of insect physiology. New York: E. P. Dutton & Co., Inc.

SOME BLOOD CONSTITUENTS OF THE NORMAL SKATE[1]

(Eleven figures)

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IN PREPARATION for a study of the chemical changes produced by interrenalectomy in the skate we needed to know the values for normal animals. Smith (1929) has published normal values for electrolytes and urea in *Raja laevis* and *R. diaphenes*. At Woods Hole the most satisfactory animal for our purpose was the common skate, *R. erinacea*, because it was plentiful and it could be interrenalectomized without too much difficulty. Little or no data have been published on the electrolytes and urea in this species. Moreover, too little attention to the effect of inanition has been given. Kisch (1930) has shown that starvation causes a fall in blood urea, but he did not determine the electrolytes.

We studied the range of variation in a large number of animals in both blood and perivisceral fluid. Then the effects of inanition and feeding were followed after it was found that plasma sodium rose with the fall in blood urea. Blood sugar and volumes per cent of erythrocytes were also determined.

METHODS

All animals reported in this paper belonged to the species *R. erinacea*. They were collected by dredging, in regular fish traps, or occasionally by hook and line. Dredging possesses the advantage that the animals are likely to be in a good state of nutrition when caught. However, skates, because of their structure, are prone to injury by pressure. Therefore to avoid this in dredging it is necessary to drag slowly and empty the net frequently.

The skates were usually kept either in a large lead-lined tank or in small, shallow wooden tanks. A few were kept in glass or concrete aquaria. All were supplied with running sea water. The water for the lead-lined tank was maintained at 10°–12° C., while the temperature of the water for the other tanks was 16°–20° C.

Feeding.—It is possible to induce some skates to eat in an aquarium after they have had 3 or 4 weeks to become accustomed to their surroundings. Neither time nor space permitted us to condition animals in this manner. We resorted to forced feeding. A glass tube about 12 mm. in inside diameter and 2 feet long was used. The desired quantity of small live *Fundulus* or ground squid or clam was forced into the tube by means of a wooden rod. Then the tube was inserted as far as the bottom of the stomach with as little struggling of the fish as possible. The contents were ejected from the tube with the wooden plunger. In this manner from 3 to 6 *Fundulus* or 15–20 cc. of ground squid or clam were introduced at one time. The animals were fed every 3 or 4 days. Sometimes

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[1] Aided by grants from the Committee on Research in Endocrinology of the National Research Council and the Ohio State University Comly Fund.

the food was regurgitated within a few hours. If this was discovered, feeding was repeated.

Blood was drawn either from the heart or from the tail by gentle suction through a 20-gauge needle. The animal was fastened, back down, on a padded concave board. Running water entered one spiracle through a rubber tube. The quantity of blood drawn at one time was most often 2.5–3.0 cc., occasionally 5 cc. Blood sugar was determined on this blood, which was collected without anesthetic and might on this account be affected by stimulation.

TABLE 1

PLASMA ELECTROLYTES IN NORMAL SKATES (*Raja erinacea*)

(Samples obtained within 24 hours after removal from the sea)

No.	Weight (Gm.)	Sodium (mEq./l.)	Potassium (mEq./l.)	Calcium (mEq./l.)	Magnesium (mEq./l.)	Chloride (mEq./l.)	Urea (mM/l.)	Erythrocytes (Vol. Per Cent)	Glucose (Mgm. Per Cent)
Male:									
44	3,400	241	5.7	11.4	6.1	252	275	18.5	58
62	3,700	244	11.9	10.6	2.0	263	343	22.0	....
65	3,800	254	11.7	9.8	1.4	267	347	22.0	73
66	3,150	260	10.0	11.2	2.8	265	322	23.0	61
68	2,625	245	6.0	12.4	.	248	335	20.0	52
87	1,975	266	6.0	13.4	1.8	268	328	20.0	62
88	2,100	246	8.9	11.2	5.0	270	308	18.0	58
Female:									
43	2,050	231	8.1	11.8	4.2	250	355	22.0	37
46	2,425	244	7.9	11.9	5.0	246	380	20.0	34
50	3,100	262	7.8	13.6	5.6	259	302	17.0	65
60	3,875	266	4.8	11.3	3.0	236	342	18.5	....
61	3,900	240	6.1	11.2	2.7	240	312	17.5	....
63	1,475	265	8.1	12.3	2.0	258	342	21.0	57
64	1,350	243	10.1	11.2	4.0	261	305	24.0	71
67	3,100	230	8.2	12.0	2.7	245	350	18.0	65
69	3,100	246	7.3	12.0	......	279	332	15.0	38
70	2,825	287	7.8	. .	.. ..	263	332	20.0	55
71	2,450	272	5.7	12.4	. .	253	343	18.0	53
85	1,600	256	6.4	11.8	6.2	254	316	18.0	38
86	3,800	263	8.9	13.8	5.9	258	303	20.0	63
89	3,850	256	11.0	14.2	3.9	246	321	19.0	75
90	2,425	252	6.0	13.0	3.5	253	323	17.0	50
92	1,700	230	11.2	......	.	250	327	......	50
94	3,275	258	7.9	......	. .	277	315	15.5	46
97	1,400	237	6.9	...	....	254	328	20.0	33
98	1,525	251	7.6	....	......	263	327	17.5	28
99	1,800	241	8.9	......	.....	240	328	19.5	55

Chemical determinations were made according to the following methods: sodium, Butler and Tuthill (1931); potassium, Consolazio and Talbott (1938); calcium, Halverson and Bergeim (1917); magnesium, Briggs (1922); chloride, Peters and Van Slyke (1932); urea, Van Slyke aeration method; glucose, Somogyi's modification of the Shaffer-Hartmann method (1937); erythrocyte volume per cent, Wintrobe (1933).

RESULTS

The samples on which these determinations were made were taken from animals which were in good condition as indicated by appearance and activity. Some were recently caught while others had been kept for variable periods. More than 100 animals were used. The animals varied greatly in size as shown in Tables 1 and 2.

The different electrolytes for the same animal are shown in Table 1. No close correlation can be found between these values. The sodium appeared to vary independently of the chloride or of the urea. Despite this, it will be shown later that plasma sodium varies inversely with the blood urea in the same individual.

TABLE 2

ELECTROLYTES IN NORMAL SKATES

No.	Weight (Gm.)	Sodium (mEq./l.)		Potassium (mEq./l.)		Calcium (mEq./l.)		Magnesium (mEq./l.)		Chloride (mEq./l.)		Urea (mM/l)	
Male:													
40	3,550	*241*[*]	236[†]	*9 3*	8 1	*10 6*	6 7	*3 9*	42 0	*255*	303	*340*	374
65.....	3,800	*254*	234	*11.7*	13.4	*9 8*	5 8	*1 4*	18 9	*267*	300	*347*	349
66.....	3,150	*260*	264	*10 0*	12.7	*11 2*	6.6	*2 8*	19 8	*265*	298	*322*	367
76 ...	3,800	*288*	264	*9 5*	12 5	*13 8*	4 6	*2 5*	30 0	*277*	262	*254*	325
79 · ··	2,575	*267*	254	*6 6*	9 0	*11 2*	8 6	*4 3*	27 0	*250*	239	*297*	330
87.. ..	1,975	*266*	318	*6 0*	11.8	*13 4*	7 8	*1 8*	19 6	*268*	260	*328*	353
88... .	2,100	*246*	246	*8 9*	13 0	*11 2*	7 2	*5 0*	22 5	*270*	272	*317*	346
Female:													
67	3,100	*230*	202	*8.2*	10 9	*12 0*	12 2	*2 7*	28 7	*249*	308	*349*	294
73	2,500	*244*	240	....	6 7	*13 4*	11 4	*5 2*	27 0	*253*	276	*334*	367
77.....	3,500	*259*	245	*7.3*	8.2	*12 4*	7 4	*6 5*	28 0	*259*	306	*337*	327
78.....	1,650	*249*	230	*6.3*	14.2	*12.4*	7 0	*2.8*	25 0	*258*	283	*322*	349
80 ...	1,575	*251*	250	*4 3*	8 1	*11 8*	10 5	*3 8*	23 0	*238*	303	*352*	330
86....	3,800	*263*	201	*8 9*	11 9	*13 8*	17.4	*5 9*	25 0	*258*	297	*303*	349
89.....	3,850	*256*	219	*11 1*	8 7	*14 2*	17 2	*3 9*	14 2	*246*	323	*321*	371
90.....	2,425	*252*	231	*6 0*	9 9	*13 0*	10.7	*3.5*	22.0	*253*	239	*323*	348

[*] Italic figures indicate plasma and blood urea.

[†] Perivisceral fluid.

A comparison of the plasma with the perivisceral fluid was made in 41 individuals. No remarkable difference was shown in the sodium values in the two fluids. Although the 15 animals chosen at random from this group for Table 2 happened to show some difference, this was not so for the whole group.

Potassium was more concentrated in the perivisceral fluid in 32 out of 37 cases. Calcium was less concentrated in the perivisceral fluid in 16 out of 22 cases. Magnesium was much more concentrated in the perivisceral fluid in all cases. Chloride was greater in the perivisceral samples in 34 out of 41 cases. Urea was greater in the perivisceral fluid in 20 out of 28 cases. Thus, in general, it may be said that magnesium, potassium, chloride, and urea were more concentrated in the perivisceral fluid, while calcium was less concentrated.

It should be noted that the perivisceral fluid varied greatly in volume. Sometimes there was too little for analysis; then again the cavity might be so gorged that the fluid welled out on puncture. No relation was observed between the volume of the fluid and the concentration of urea and electrolytes. A comparison of the values for each substance was made in the blood or plasma of many different individuals.

Sodium.—The distribution of plasma sodium is shown in Figure 1. The extremes were 219 and 289 mEq./l. The mean value was 254 mEq./l. Two-thirds of the values lay between 240 and 270 mEq./l. The range shown in samples taken from fish within 24 hours after removal from the sea is practically the same as that for fish kept over a longer period.

Sodium distribution in the perivisceral fluid shows a much wider range. The lowest values were 201 and 202, and the highest were 304, 318, and 463 mEq./l. These are not shown in the figure.

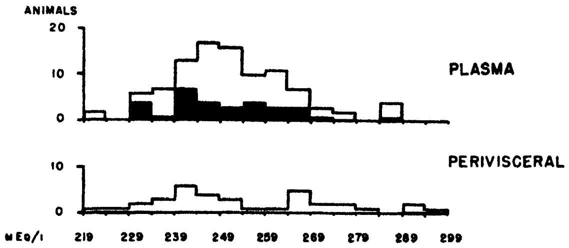

FIG. 1.- Distribution of sodium in the skate. Those tested within 24 hours of removal from sea shown in black.

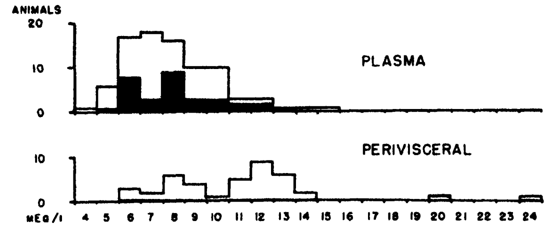

FIG. 2.—Distribution of potassium in the skate. Those tested within 24 hours of removal from sea shown in black.

Potassium.—Hemolysis occurs rather easily in skate's blood, making the potassium values high. Whenever there was evidence of hemolysis the potassium determinations were discarded.

Plasma potassium ranged from 4 to 15 mEq./l., with the mean at 8. The range of the values in animals sampled within 24 hours after removal from the sea was not quite so great. In perivisceral fluid, potassium ranged from 6 to 24 mEq./l. with the mean at 10. Thus there was a tendency for potassium to be more concentrated in the perivisceral fluid (Fig. 2).

Calcium.—Plasma calcium lay between 6 and 14 mEq./l., with a mean of 12. The perivisceral calcium ranged from 3 to 18 mEq./l. (Fig. 3). Generally speaking, it was less concentrated than in the plasma.

Magnesium.—Plasma magnesium ranged between 2 and 7 mEq./1., with a mean of

5 (Fig. 4). The perivisceral magnesium concentration varied so greatly that it could not be charted conveniently. It extended from 6 to 98 with a mean of 42 mEq./l.

Chlorides.—The plasma chlorides ranged from 230 to 285, while the mean was 255 mEq./l. (Fig. 5). Samples taken not more than 24 hours after capture of the fish gave a similar range. The range in the perivisceral fluid was greater, being from 235 to 330 for all except two samples not shown in the chart, which were 385 and 400 mEq./l. The mean was 300 mEq./l., which is a very definite shift toward greater concentration.

Urea.—The blood urea ranged from 254 to 384 mM/l., with a mean value of 320 (Fig. 6). The range was not so great in animals tested within 24 hours of removal from the sea. The small series from perivisceral fluid showed a possible shift toward greater concentration.

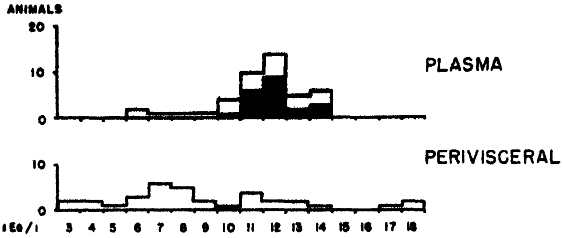

FIG. 3

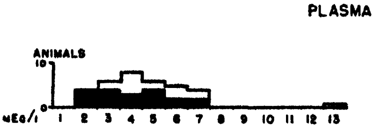

FIG. 4

FIG. 3.—Distribution of calcium in the skate. Those tested within 24 hours of removal from sea shown in black.

Fig. 4.—Distribution of magnesium in the skate. Those tested within 24 hours of removal from sea shown in black.

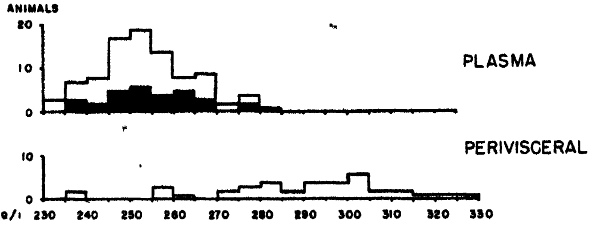

FIG. 5.—Distribution of chloride in the skate. Those tested within 24 hours of removal from sea shown in black.

Erythrocytes.—The volume per cent of erythrocytes ranged from 11 to 24, with the mean at 18. In animals sampled within 24 hours of removal from the sea the lowest value was 15 and the mean was 19 (Fig. 7).

Blood glucose.—The blood glucose ranged from 0 to 105 mgm. per cent, with a mean of 45. In fish tested within 24 hours of removal from the sea the range was 30–75 mgm., with a mean of 54 mgm. per cent (Fig. 8).

Recapitulating, our results indicate that skates recently removed from the sea have a smaller range of blood urea and blood sugar and a higher volume per cent of erythrocytes than do skates removed for a longer period. Noteworthy are the higher values of potassium, urea, magnesium, and chloride, especially the last two, in the perivisceral fluid as compared to those in plasma.

The differences observed between tests made on skates within 24 hours after their removal from the sea and those made after a longer period led us to study the effects of inanition and feeding.

Effect of inanition.—The effect of inanition was studied in 10 skates. Six of them probably started the experiment as fed animals, because they were used within 24 hours after dredging them from the sea. Evidence that this was so was furnished by 7 skates killed immediately after removal from the sea. In all cases the alimentary canal contained food in some stage of digestion. Food was present in the stomach and spiral valve of all but 1, which contained food only in the spiral valve. Blood samples were drawn from the 6 animals (Nos. 45, 46, 51, 62, 123, and 44 [Fig. 9]) after 7 days' starvation. All showed considerable increase in plasma sodium. The blood urea was decreased in all but 1 (No. 44). This decrease was considerable in 4

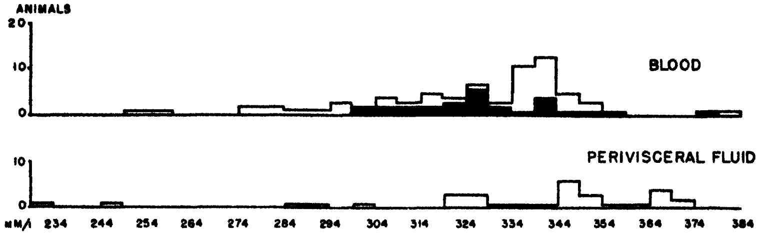

FIG. 6.—Distribution of urea in the skate. Those tested within 24 hours of removal from sea shown in black.

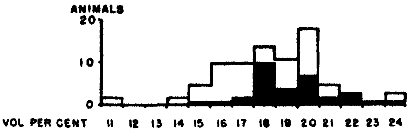

FIG. 7

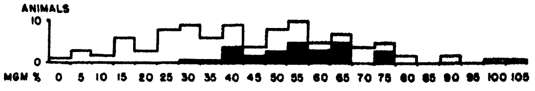

FIG. 8

FIG. 7.—Distribution of erythrocyte volume per cent in the skate Those tested within 24 hours of removal from sea shown in black.

FIG. 8.—Distribution of blood sugar in the skate. Those tested within 24 hours of removal from sea shown in black.

Four additional animals (Nos. 60, 61 122, and 124 [Fig. 9]), which had been kept for several days before the experiment was started, were observed for longer periods. They showed similar but more gradual changes, plasma sodium increasing and blood urea decreasing.

Plasma chloride was increased at the same time that the sodium rose. The values were as follows: **44**, 252, 278; **45**, —, 282; **46**, 246, 279; **51**, 270, 274; **60**, 236, 307; **61**, 240, 266, 278; **62**, 263, 284; **122**, 233, 236, 252, 258, 264, 314; **123**, 258, 260; **124**, 240, 250, 254, 260, 264, 326 mEq./l.

Plasma calcium was determined in 7 of the skates. It ranged from 11 to 13 mEq./l. and showed little or no change during inanition.

Plasma magnesium was determined in 5 skates. It showed an increase with inanition. The values were as follows: **44**, 6.1, 9.2; **51**, 4.9, 6.0; **60**, 3.0, 6.6; **61**, 2.7, 4.0, 4.3; **62**, 2.0, 2.3 mEq./l.

In sugar determinations there was considerable variation, due in part, no doubt, to the stimulation attendant upon obtaining the blood. However, later samples were often higher than early ones. The values corresponding to the same time intervals and in the same order as shown in Figure 9 for the electrolytes were as follows: **44**, 58, 75, 68; **45**, 43, 70; **46**, 34, 23; **60**, —, 18, 25; **61**, —, 25, 77; **122**, 43, 25, 50, 80, 112, —; **123**, 68, 67; **124**, 30, 27, 40, 64 mgm. per 100 cc.

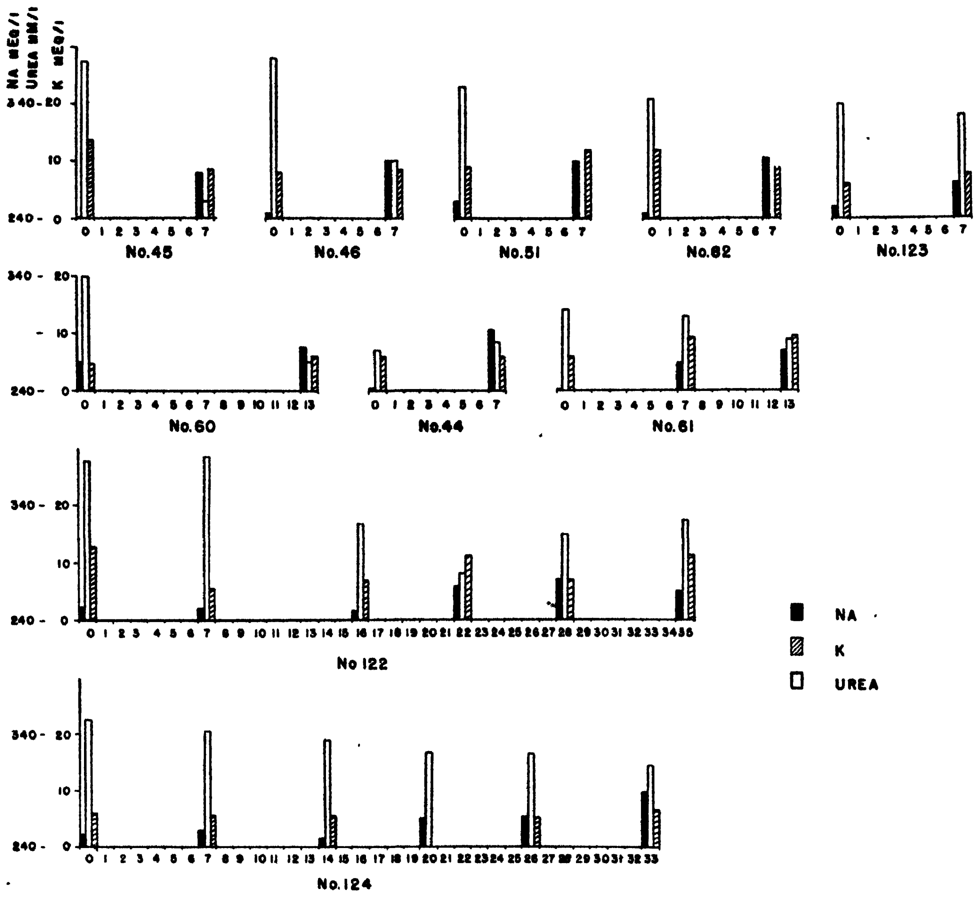

FIG. 9.—Blood changes during inanition

The volume per cent of erythrocytes was also determined when other determinations were made, in all animals except Nos. 45 and 123. In only 1 animal was there more than a 12 per cent change, usually a fall. The values were as follows: **44**, 18.5, 16.5, 16.5; **46**, 20.0, 18.0; **60**, 18.5, 15.0, 13.0; **61**, 17.5, 18.0, 16.5; **122**, 17.0, 16.0, 17.5, 18.0, 18.0; **124**, 16.0, 16.0, —, 18.0 volume per cent.

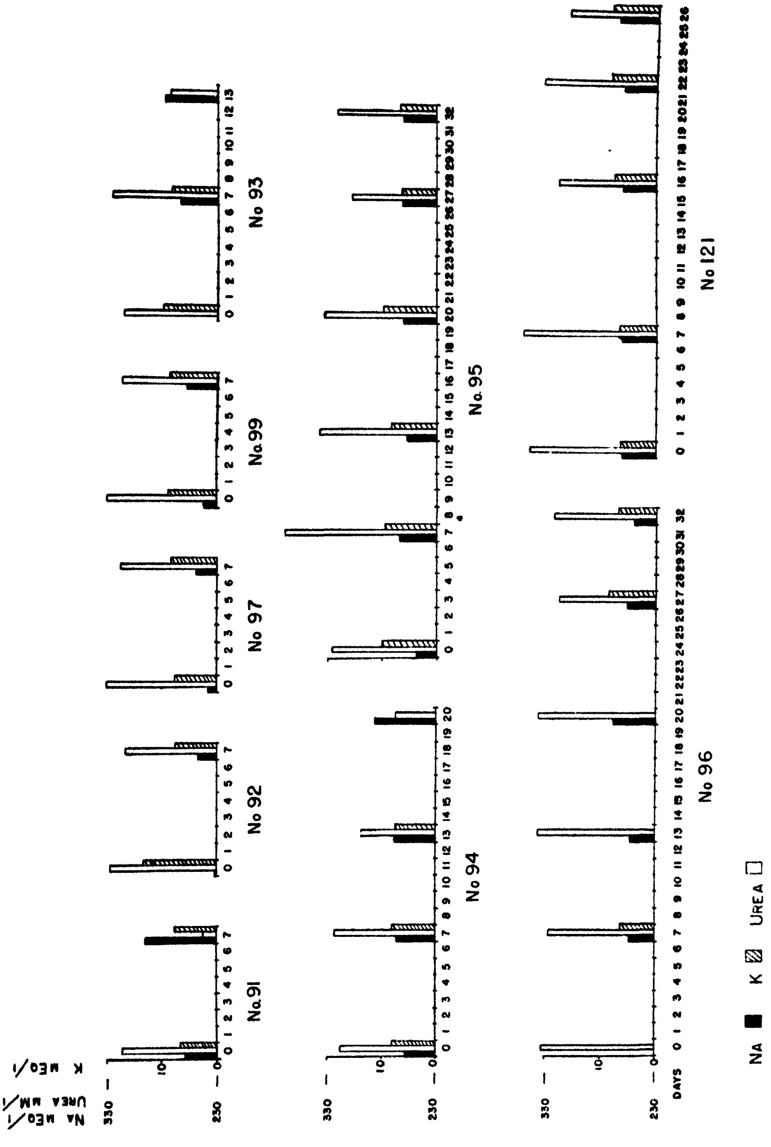

FIG. 10.—Blood changes in fed animals

It should be stated that only 2 of the animals (Nos. 122 and 124) were killed, the others dying 3–7 days after the last sample was taken.

Thus it is seen that the typical changes in inanition are a fall in blood urea and a rise in plasma sodium, chloride, and possibly magnesium. In order to prove that these changes were due to inanition, another series of skates were kept under similar conditions but fed as described earlier.

Effect of feeding.—Nine skates were fed for periods of time ranging from 7 to 32 days. Three of them showed a decided drop in blood urea (Fig. 10, Nos. 91, 93, and 94). The others showed a minor reduction. However, in general it could be said that these changes were not so great as in the unfed animals. The plasma sodium increased as in inanition but to a lesser degree.

Plasma chloride was more variable, increasing in some animals but showing little change in others. The values were as follows: **91**, 256, 307; **92**, 250, 276; **93**, —, 242, 260; **94**, 277, 268, 256, 286; **95**, 253, 254, 250, 261, 256, 256; **96**, 266, 257, 242, 273, 257, 251; **97**, 254, 230; **99**, 240, 263; **121**, 245, 248, 264, 249, 255 mEq./l.

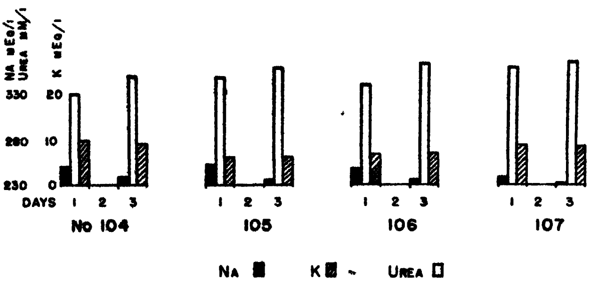

FIG. 11.—Effect of urea injection on plasma sodium

Blood sugar varied as in the starved animals. The values were as follows: **91**, 52, 68; **92**, 50, 38; **97**, 33, 55; **99**, 55, 54; **93**, 26, 33; **94**, 46, 103, 143, 177; **95**, 51, 37, 26, 18, 18, 25; **96**, 27, —, 28, 30, 28, 37; **121**, 56, 40, 31, 54, 55 mgm. per 100 cc.

The volume per cent of erythrocytes showed little change. The values in 6 skates were as follows: **91**, 18.0, 16.5; **92**, 15.0, 14.5; **97**, 20.0, 21.0; **99**, 19.5, 21.0; **93**, 16.0, 16.5, 16.5; **94**, 15.5, 14.5, 15.0, 16.0; **95**, 19.0, 17.0, 17.5, 16.5, 15.0, 15.0; **96**, 15.0, 15.0, 15.5, 14.0, 14.0, 14.5; **121**, 18.0, 14.0, 14.0, 15.0 vol. per cent.

Three of the animals were killed or used for other investigations. In spite of the feeding, the others died in from 3 to 7 days after the last determinations were made.

It can be seen from the above results that the fall in blood urea and rise in plasma sodium are in the same direction as in inanition but that they are less marked. Therefore, inanition appears to be a causative factor.

A fall in blood urea is accompanied by a rise in plasma sodium. If it could be shown that a rise in urea is accompanied by a fall in plasma sodium, the change in urea concentration would appear to be responsible for the changes in sodium concentration. Experiments were undertaken to demonstrate this point.

Effect of urea injection on plasma sodium.—The effect of blood urea concentration on plasma sodium was determined by the injection of urea into 4 normal skates, weighing between 975 and 1,450 gm. They had been caught 6 days before beginning the experiments. The injections were made daily into the muscle of the tail. A 25 per cent solution of urea was employed. Two animals received 1 cc. per kilo body weight, and 2 received double this dose. The injections were continued for 3 days. Blood samples were taken just before the first injection and 24 hours after the last injection.

The blood urea was increased, as would be expected, while the plasma sodium was decreased. The double dose of urea produced no greater effect than the smaller dose. The significant point is that the plasma sodium fell with the rise in blood urea in all experiments. The results are shown in Figure 11.

DISCUSSION

The relatively short survival of many skates under laboratory conditions is difficult to explain. The various factors concerned might be lack of food, temperature, pressure, injury, infection, or intoxication.

Simpson and Ogden (1932) found that dogfish died if the temperature rose above 18° C. Although most of our animals were maintained at 10°–12° C., some that survived the longest were kept at 16°–20° C. Therefore, temperature was not a major factor. Undoubtedly, inanition was a factor in unfed animals, but many fed animals survived no longer than those unfed. However, the feeding was inadequate because all fed animals lost weight. More frequent feeding was indicated. Reduced pressure might have contributed to the short survival. Skates are usually found at depths of 3 meters or more. The water in our tanks was 0.6 meter deep or less. Change in pressure itself could scarcely be responsible, except that it may have contributed to the discontent shown by many animals. Occasionally, undetected injury might have occurred when the fish were caught. There was no reason to believe that the bacterial pollution of the water which supplied the aquaria was sufficient to be a factor, although sewage was present in the general region from which the water was pumped. We found no evidence that the type of aquarium was a factor in the survival. Frequent attempts to escape indicated that many of the skates were disturbed by the confinement.

It will be noted that some of our animals were used after they had been in the laboratory for several days. This did not seem to vitiate the experiment, because the results were comparable with those obtained from freshly caught skates.

The observation that sodium and urea concentrations in the plasma tend to reciprocate each other in the same individual supports the idea that urea plays a part in osmotic regulation (Smith, 1936). Although feeding retarded the fall in urea, it did not prevent it. This would indicate that the amount of food was inadequate.

Para (1936) studied the effect of successive bleedings on the blood of the skate. He bled as many as six times at intervals of a few days, removing 30 cc. each time. His animals weighed 4–6 kgm. We never removed more than 5 cc. at one time, usually 3 cc. or less. In other words, in proportion to the body weight he removed about five times as much blood as we did. He found that the serum chloride increased after several days. This increase must have been due to inanition as well as to loss of urea contained in shed blood.

The great range in blood glucose is very striking. The higher values might be caused by stimulation associated with sampling. It is interesting that such low blood sugar values were found in apparently normal animals.

We confirmed Smith (1929) in finding that the calcium of the perivisceral fluid was somewhat less than that in the plasma. We differ from him in finding that the concentration of potassium in perivisceral fluid was somewhat greater than that in the plasma. Perivisceral fluid chloride, likewise, was somewhat greater than that in plasma.

The difference in concentration between substances in the perivisceral fluid and the blood indicates that the blood vessels exercise a selective influence on exchanges with the perivisceral fluid. The greater concentration of potassium, urea, magnesium, and chloride in the perivisceral fluid would indicate their excretion from the blood via this route. The abdominal pores make it possible for the perivisceral fluid to escape from the body.

SUMMARY

Plasma electrolytes, urea and sugar in the blood, and electrolytes and urea in perivisceral fluid were determined in a large number of skates belonging to the species *R. erinacea*. The concentrations of potassium, magnesium, chloride, and urea were greater and that of calcium less in perivisceral fluid than in plasma. Skates recently removed from the sea showed a smaller range of blood urea and blood sugar and a higher volume per cent of erythrocytes than those removed for a longer period. During inanition blood urea fell while plasma sodium, magnesium, and chloride increased. The volume per cent of erythrocytes tended to decrease. Feeding retarded these changes. A rise in blood urea from injection of this substance caused a fall in plasma sodium. Thus concentrations of urea and sodium tend to be complementary.

We wish to thank Dr. C. M. Osborn of the Woods Hole Oceanographic Institute for dredging facilities and Mr. R. A. Goffin for the facilities of the United States Bureau of Fisheries.

LITERATURE CITED

BRIGGS, A. P. 1922. A colorimetric method for the determination of small amounts of magnesium. Jour. Biol. Chem., 52:349–355.

BUTLER, A. M., and Tuthill, E. J. 1931. An application of the uranyl zinc acetate method for determination of sodium in biological material. Jour. Biol. Chem., 93:171–180.

CONSOLAZIO, W. V., and TALBOTT, J. H. 1938. Modification of the method of Shohl and Bennett for the determination of potassium in serum and urine. Jour. Biol. Chem., 126:55–61.

HALVERSON, J. O., and BERGEIM, O. 1917. The determination of small amounts of calcium, particularly in blood. Jour. Biol. Chem., 32:159–170.

KISCH, B. 1930. Harnstoffuntersuchungen bei Selachiern. Biochem. Zeitschr., 225:197–207.

PARA, E. 1936. L'Influence des saignées successives sur la composition chimique et physico-chimique du sang des animaux marins. Jour. physiol. path. gén., 34:735.

PETERS, J. P., and VAN SLYKE, D. D. 1932. Quantitative clinical chemistry, 2:834. Baltimore: Williams & Wilkins.

SIMPSON, W. W., and OGDEN, E. 1932. The physiological significance of urea. I. The elasmobranch heart. Jour. Exper. Biol., 9:1–5.

SMITH, H. W. 1929. The composition of the body fluids of elasmobranchs. Jour. Biol. Chem., 81: 407–419.

SMITH, H. W. 1936. The retention and physiological role of urea in the elasmobranchii. Biol. Rev., 11: 4–82.

SOMOGYI, M. 1937. A reagent for the copper-iodimetric determination of very small amounts of sugar. Jour. Biol. Chem., 117:771–776.

WINTROBE, M. M. 1933. Macroscopic examination of the blood. Amer. Jour. Med. Sci., 185:58.

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